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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

A. G. DuMez, Editor, Baltimore, Maryland

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Quantitative Aspects of Biological Assay

*By C. I. Bliss**

A biological assay may be defined as any determination of toxicity or potency based upon the reaction of living matter. In a broader sense it covers research on new drugs but here we are concerned with the standardization of specific extracts, hormones, vitamins, sera, etc., where the concentration of active agent cannot be analyzed chemically. While the inherent precision of biological indicators is considerably less than that obtainable in analytical chemistry, this precision is measurable and potency can be estimated within any required limits of error.

Certain common factors underlying good assay technique may be listed in five categories.

(*a*) There should be substantial equivalence between the activity of the drug in a test animal and in man, so that samples producing the same reaction in the laboratory will have the same effect therapeutically. Since the biologically-standardized drugs often contain either impurities or several active principles in a complex mixture, two samples giving equal reactions in one species may give different reactions in another species. Assays in saline solution of crystalline standard insulin in comparison with the original impure standard gave an average potency some 19 per cent greater by the mouse method than by the rabbit, as shown in Table I. Similarly, Gold and Kwit (13)

Table I.—Assay of Crystalline Standard Insulin in Terms of the Original Impure Standard. Tests in Saline Solutions (14)

Laboratory	Units per mg. Rabbit Method	Units per mg. Mouse Method
Toronto		
(Hershey and Lacey)	21.3	24.8
(Scott)	....	24.0
England		
(A. B. Insulin Laboratories)	21.9	28.0
(Wellcome Laboratories)	....	27.0
Copenhagen	....	25.4
Indianapolis	21.9	....
Unweighted mean	21.7	25.8

have observed a three-fold difference in the therapeutic dose for man for preparations of digitoxin and of ouabain assayed as equipotent by the cat method. This factor deserves more careful study but is beyond the scope of the present report.

(*b*) The reaction should be sensitive to relatively small changes in dose and the characteristic curve relating these two terms should be determined experimentally. Assays are ill-contrived which depend upon an assumed direct proportionality between dosage and response, as in the U. S. P. XI parathyroid assay. The observed relation between the increase in the serum calcium

* Sandusky, Ohio

of the dog and the log-dose of parathyroid extract has been plotted from the data of Lloyd C. Miller (15) in Fig. 1, after adjustment for differences between dogs (6). A comparison of the computed curve with that defined by the U. S. P. XI monograph on parathyroid extract illustrates the discrepancy that can arise in the case of a graded response. When based upon an all-or-none

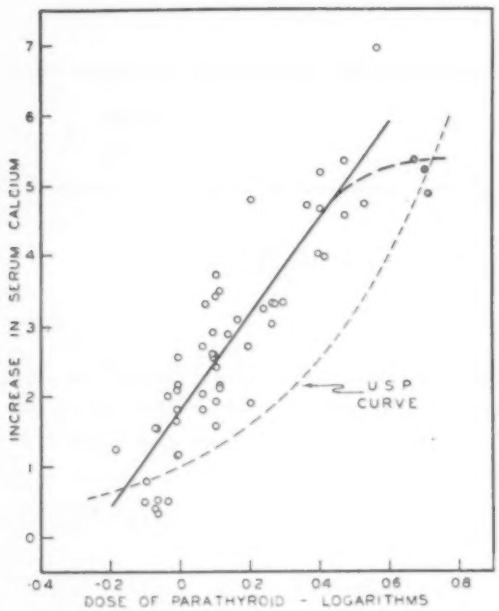

Fig. 1.—Dosage-Response Curve for Parathyroid Extract in Terms of the Increase in Serum Calcium of Dogs, Data of Lloyd C. Miller (6, 15), Showing the Discrepancy between the Observed Relation and That Assumed by the Pharmacopœial Monograph.

reaction, the characteristic curve relating dosage of drug and percentage effect is sigmoid and asymmetrical. A typical dosage-effect curve is that for the toxicity of ouabain to frogs as reported by Chapman and Morrell (8) and plotted in Fig. 2.

Normally the general form of the characteristic curve can be established with a single preparation of the drug. But the average or overall susceptibility of the test animals, which fixes the position of the curve, and to a lesser extent their sensitivity to a change in dose, determining the slope of the curve, tend to vary from one laboratory to another and from one time to another in the same laboratory. In the experiments behind Fig. 2, eight different "standard" curves were determined within a period of three weeks, each from 240 frogs in the same laboratory. Yet the individual curves differed significantly both in position as measured by the LD50 or its logarithm and in slope or b as computed from the transformed coördinates (Table II). Because of this

Table II.—Variations in "Standard" Dosage-Effect Curves for Ouabain. Each Curve Determined from 8 Groups of 30 Frogs by Over Night Technique, Computed in Terms of Log-Dose and Probit Mortality, Data of Chapman and Morrell (8)

Date, 1930	$LD50^{-1}$ per Gm. of Frog	Log-LD50	Slope b
Aug. 26	32.42 ±0.10	1.5108 ±0.0014	25.9 ±2.7
Aug. 28	28.51 ±0.39	1.4550 ±0.0060	15.3 ±2.1
Sept. 2	27.03 ±0.11	1.4319 ±0.0017	22.8 ±2.9
Sept. 4	30.54 ±0.10	1.4849 ±0.0014	24.9 ±2.5
Sept. 8	31.68 ±0.15	1.5008 ±0.0020	17.6 ±2.0
Sept. 10	32.03 ±0.12	1.5056 ±0.0017	22.2 ±2.8
Sept. 12	31.27 ±0.11	1.4951 ±0.0015	22.1 ±2.2
Sept. 16	31.21 ±0.10	1.4943 ±0.0014	27.8 ±2.9

variability, the curves on August 28th and on September 8th were omitted altogether and the remaining six curves adjusted for differences in LD50 in preparing Fig. 2. An erratically low mortality of 73 per cent was also omitted in computing the curve but it is included in the diagram. The essential relation between reaction and dose should be

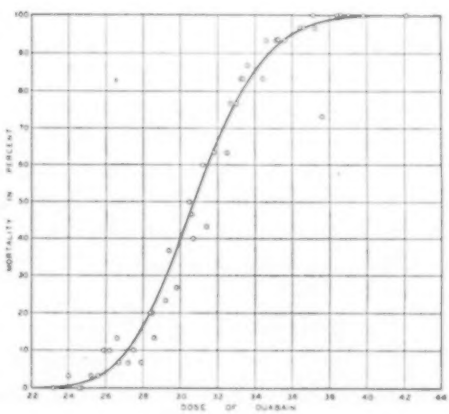

Fig. 2.—Dosage-Effect Curve for Ouabain in Frogs with Each Point Based upon a Separate Lot of 30 Frogs, Data of Chapman and Morrell (8). Six Different "Standard Curves" Have Been Adjusted to a Mean Level of Susceptibility to Facilitate Plotting Them in a Single Diagram.

determined, therefore, as an integral part of any self-contained assay. This is greatly facilitated where the reaction can be plotted as a straight line against the logarithm of the dose, so that an important objective in

analyzing the characteristic curve is to discover which criterion of effect best accomplishes this purpose.

(*c*) The unit of potency should be defined in milligrams of a stable reference standard of the drug and not in terms of biological effect. The concentration of each sample or unknown is adjusted so that it will produce quantitatively the same reaction as the standard when tested in parallel. This eliminates large, erratic differences caused by seasonal and secular change, by inherent and environmental differences between colonies of laboratory animals and by variations in manipulative detail. Susceptibility may change over such short intervals of time that the standard is included in each assay to correct differences similar to those shown in the first columns of Table II. There remain principally the unavoidable variations in sensitivity between the individuals of any given assay.

Although the concentration of an unknown can be adjusted by trial and error alone until it produces the same reaction as the standard, it is preferable to utilize rather than to ignore the underlying curve relating dosage and response. Determined as part of the assay, this curve enables the experimenter to convert an observed difference in the reactions to standard and to unknown to units of dosage and thence to relative potencies.

(*d*) Assays should be designed so that doses of the unknown and of the standard are administered under as nearly identical conditions as are practicable. By identifying unavoidable sources of variation and balancing them equally between the two samples of drug and the different concentrations of each, bias can be avoided and the experimental error reduced. The increase in precision with an effective design is illustrated by an experiment on the rabbit assay of insulin reported by Bliss and Marks (5). Here each of eight rabbits was given four different doses of insulin on four different days as determined by two Latin squares and the hypoglycæmic response measured from the mean percentage fall in blood sugar over a five-hour period. The different sources of variation have been segregated and appraised in Table III by the analysis of variance. By "quarantining" differences in susceptibility between individual rabbits and between days, they neither biassed the comparison between doses nor contributed to the estimate of experimental error. The variance ratios in the last column of Table III, computed by dividing the "mean squares" for each of the first three factors by that for experimental error, show that individual differences in susceptibility between rabbits and between days were many times larger than the experimental error, from which these terms had been removed, with a corresponding accentuation of the contrast between the dosage factor and the error.

Table III.—Partition of the Sources of Variation by the Analysis of Variance in an Experiment on the Rabbit Assay of Insulin, from Bliss and Marks (5)

Variation Due to	Degrees of Freedom	Sum of Squares	Mean Square or Variance	Variance Ratio (F)
Differences between rabbits	7	1702.20	243.17	8.28
Differences between days	3	403.72	134.57	4.58
Differences between doses	3	2330.58	776.86	26.45
Experimental error	18	528.68	29.371	1
Total	31	4965.18		

(*e*) Finally a biological assay should provide an objective measure of its experimental error as an integral part of the determination of potency. Even under well defined conditions assays vary in their inherent precision, which we measure in terms of the limits within which the true potency of the unknown may be expected to fall for any given odds. The Subcommittee on the Accuracy of Biological Assays for the British Pharmacopœia has determined the expected variation in the potency of certain drugs when the observed response is the same on both the standard and the unknown. In one official assay in 100 or at $P = 0.01$, the true potency is expected to exceed the limits given for the five selected assays in Table IV for an apparent potency of 100 per cent.

Table IV.—Average Limits of Error (P = 0.99) of Selected Biological Assays in the British Pharmacopœia (7); the Response Assumed Equal on Unknown and on Standard

Vitamin A (5 weeks' growth)	37–272 per cent
Vitamin C (lesions of scurvy)	82–139 per cent
Vitamin D (line test)	49–215 per cent
Staphylococcus antitoxin (intravenous injection in mice)	92–108 per cent
Antipneumococcus serum, Type I (injection of mixtures of serum and culture)	57–176 per cent

It is evident that official assays varied widely in their precision. These average limits of error were based upon equal reactions to standard and to unknown and frequently would be larger due to a difference in the reaction. Since individual assays or the general level of precision in a given laboratory may differ considerably from the average limits shown in the table, an integral part of each assay should be a determination of its experimental error. Hence the data for computing this error should be obtained routinely in a good determination of relative potency.

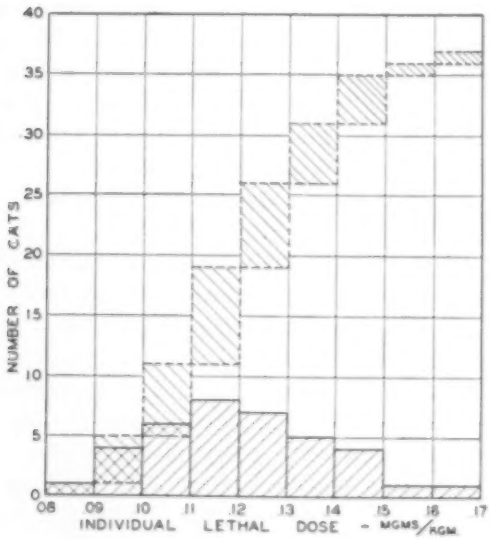

Fig. 3.—Frequency Distribution of the Individual Toxic Doses of Ouabain in 37 Cats, Data of Chen, Chen and Anderson (9), Plotted Directly at the Base of the Diagram and above It as a Cumulative Curve.

The application of concrete statistical procedure to problems of biological assay depends in part upon the nature of the reaction used to measure drug potency. Assays may be classified under three main headings: (1) those based upon an all-or-none reaction, (2) those based upon reaction time and (3) those depending upon a graded response.

(1) Many assays depend upon an all-or-none or qualitative reaction and an unknown is judged to have the same potency as the standard when equal percentages of test animals react characteristically to corresponding dosages of the two samples. The dosage-effect curve relating the percentage of positive reactions to the dosage of drug is typically an asymmetrical sigmoid curve, such as that shown in Fig. 2. The animals in an experiment differ in their susceptibility to the drug and the curve owes its shape to this variation. Because the variation conforms to the well-known normal distribution within the limits of experimental error, the dosage-effect curve can be converted easily to a straight line which greatly facilitates its analysis.

The statistical nature of the dosage-effect curve and its transformation can be understood most readily in terms of the cat method for assaying cardiac glucosides, where the just-toxic dose is measured directly for each individual by slow intravenous infusion. Because of individual differences in susceptibility, the required amount of drug varies from one cat to another even after adjustment for differences in body size. A typical series is supplied by the just-toxic dose of ouabain for 37 cats as determined by Chen, Chen and Anderson (9). These have been arranged in an increasing order, grouped in nine equal dosage intervals and plotted along the base of Fig. 3, the largest number of cats occurring at an intermediate dose with the numbers diminishing at larger and smaller doses. The resulting bell-shaped curve is the familiar one descriptive of biological variation. Each block along the base of Fig. 3 was then moved vertically upward until its lower edge coincided with the upper edge of the next preceding block, transforming the curve to the cumulative sigmoid form with the number of cats dying at all doses up to and including any given amount of ouabain plotted against the dose. This curve has been replotted in Fig. 4 with the ordinate changed to percentages and is

statistically identical with Fig. 2, which describes the reaction of frogs to the same drug. The difference between them is one of experimental technique, the several points

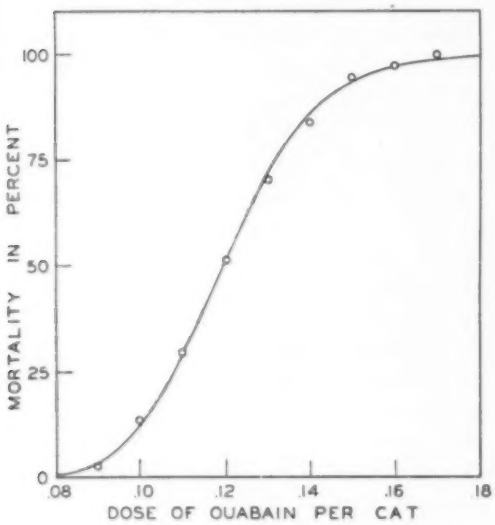

Fig. 4.—Dosage-Effect Curve for Ouabain in Cats, Computed from the Data in Fig. 3.

in Fig. 4 representing different readings on a single series of 37 cats, while each of those in Fig. 2 is the percentage reaction of a separate group of 30 frogs to a single dose of ouabain administered uniformly to each frog in the group.

The normal distribution or curve of error with which these curves are to be identified is shown in Fig. 5 in the more familiar, bell-shaped form. The "dosages" along the base are in terms of standard deviations, 0 representing the mean (M). In order to avoid negative values, it is convenient to add 5 to each standard deviation, the resulting unit being known as a "probit" (1). Of the characteristics of this well-known curve, the one of immediate interest is the relation between the probit and the proportion (p) of the total area under the curve ($p + q$) lying to the left of a perpendicular (such as xz) erected at any point (x) along the base. The proportionate area, which may be identified with the proportion or percentage killed, has been plotted against a theoretical dosage or probit in Fig. 6 and again we have a sigmoid curve paralleling those in Figs. 2 and 4. Disregarding the known dosage of ouabain, each observed percentage (or proportion) in Fig. 2 or 4 can be spotted on the theoretical curve in Fig. 6 and the corresponding probit or "thoretical dosage" read from the base. When the probit is plotted against the actual dose of ouabain in

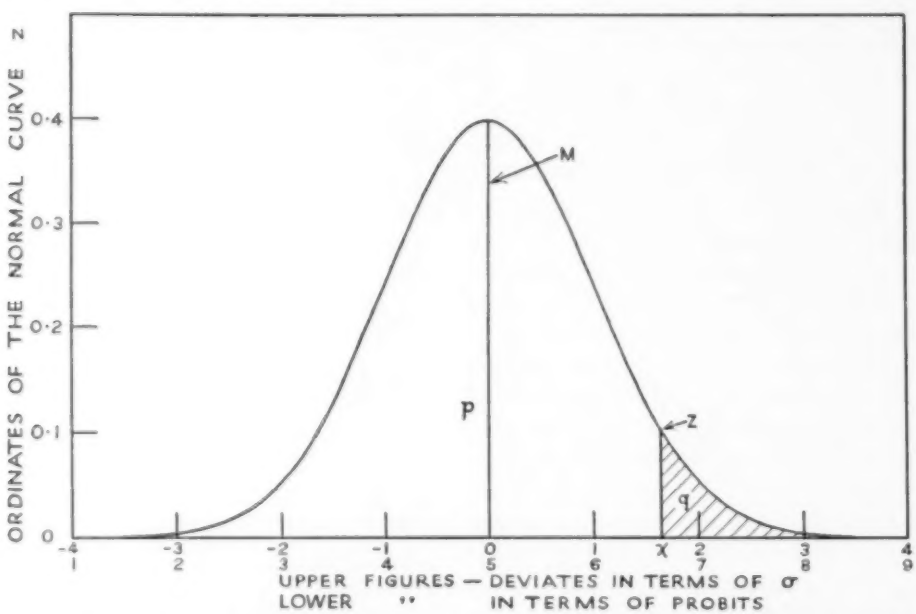

Fig. 5.—The Theoretical Normal Curve of Error, Believed to Underlie the Variation in the Susceptibility to Drugs, after Bliss (2). The Abscissa Corresponds to the Log-Dose and the Proportion of the Total Area to the Left of Any Given Vertical, Such as xz, to the Percentage Effect.

logarithmic units, the resulting diagram of inferred dose against the observed dose defines a straight line (Figs. 7 and 8). Gaddum (12) has given the curve in Fig. 6 in a form suitable for interpolating the normal equivalent deviation or "N. E. D." from the

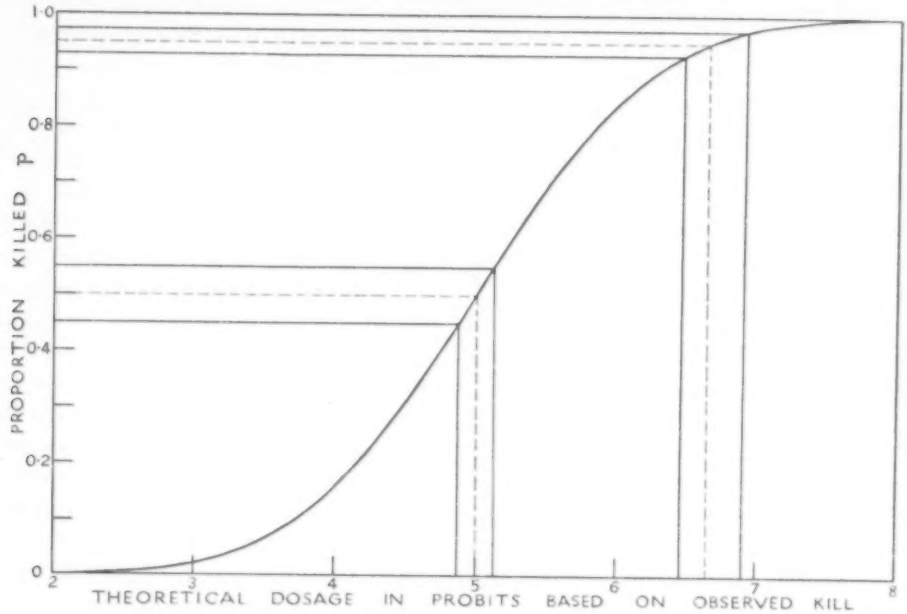

Fig. 6.—The Theoretical Normal Distribution of Fig. 5 Plotted in the Cumulative Form, from Which the Observed Percentage Reaction on the Ordinate Can Be Transformed to the Theoretical Dosage or Probit Along the Abscissa.

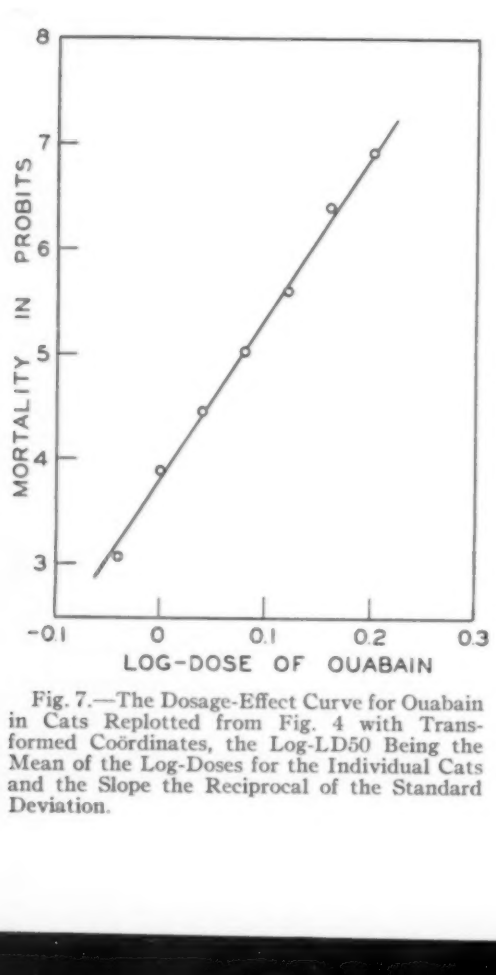

Fig. 7.—The Dosage-Effect Curve for Ouabain in Cats Replotted from Fig. 4 with Transformed Coördinates, the Log-LD50 Being the Mean of the Log-Doses for the Individual Cats and the Slope the Reciprocal of the Standard Deviation.

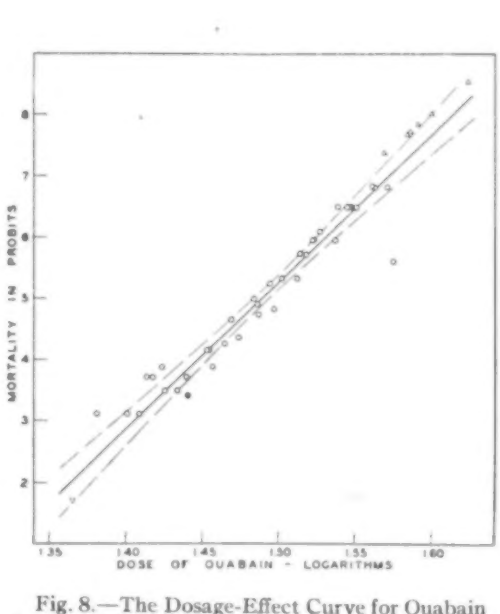

Fig. 8.—The Dosage-Effect Curve for Ouabain in Frogs Replotted from Fig. 2 with Transformed Coördinates. The True Value Lies within the Curved Lines within Odds of 99 in 100, the Individual Curves Being Corrected for Overall Variations in Susceptibility and Two out of Eight Series Omitted due to a Significantly Flatter Slope. The Isolated Record at 5.6 Probits Was Omitted from the Final Computation.

percentage effect, but it is more convenient to use specially ruled logarithmic-probability paper or a table of probits (2, 11). The more general indirect method of thus measuring the distribution of susceptibilities from the percentage reaction of separate lots of animals is that referred to as the dosage-mortality or the dosage-effect curve.

The transformation to a straight line has provided a rational interpretation of the dosage-effect curve and has shown that to obtain uniform increases in effect with most drugs the dosage must be increased not by equal increments but by a constant proportion. When in linear form, these curves can be computed so as to estimate the dosage producing any required percentage effect and the limits within which it has been determined for any given odds. The dosage-effect curve in Fig. 8, for example, as determined from six of the eight series, would not be expected to vary in slope beyond the curved lines of the diagram more than once in 100 times if redetermined from a similar number of equivalent frogs. The fact that in 2 of the 8 series the frogs were not equivalent emphasizes the desirability of so conducting assays that the slope can be checked.

By administering two or more doses of both standard and unknown, a curve can be determined separately for each and their inherent validity tested objectively. If the unknown sample produces in fact the same biological effect as the reference standard, these two straight lines should be substantially parallel, a single combined slope will fit both series of observations adequately and the estimate of potency holds at all dosage levels. Prigge's data (16) on the assay of a diphtheria aluminium antitoxin from the percentage survival of guinea pigs may be taken as an example. These have been plotted in Fig. 9 in terms of probits and logarithms. With 23 to 25 animals on each dose, the observations for both standard and unknown could be fitted adequately by parallel lines as tested by $\chi^2 = 3.86$ with 4 degrees of freedom for the assay as a whole. Then on the log-dosage scale the horizontal distance between the two parallel lines, designated by the symbol M, measures the relative potency of the unknown in terms of the reference standard. The standard error of M, s_M, is readily determinable and after adjustment for the assumed unitage of the unknown, both M and s_M can be transformed to the percentage or proportionate potency with a table of logarithms. In the example of Fig. 9, computation gave $M = 1.507 \pm 0.083$ or the aluminium antitoxin was assayed as 32.12 ± 6.15 as potent as the standard. .

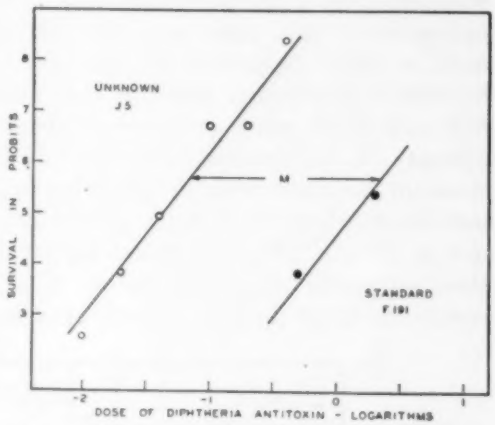

Fig. 9.—The Biological Assay of a Diphtheria Antitoxin from an All-or-None Response as Computed from Prigge's Data (16), the Horizontal Distance between the Parallel Lines (M) Being the Log-Ratio of Potencies.

(2) Occasionally the reaction time to a qualitative response varies sufficiently with the dose that it can be used as an assay criterion. In most all-or-none assays, results are scored when the reaction is substantially complete, so that a later listing would reverse comparatively few scores. Dosages are adjusted to fall within the range giving from 5 to 95 per cent response and the reaction time is noted primarily to insure that the ratings are not made before the drug has had a chance to exert its full effect or after secondary factors may have entered the picture. Although the reaction time may be relatively independent of individual susceptibility to a drug, in some cases these two aspects are linked so closely that the results from the larger doses can be scored much earlier than those from small doses. It then becomes practicable to work within a higher dosage range and to use reaction time as the index of effect.

When measuring drug action in terms of

the reaction time, the time to reach a constant end-point should be related to the dose. This does not occur when the percentage of individuals which have reacted is noted at some arbitrary interval after treatment, for both the level of effect and the speed of the reaction would contribute to such a percentage. Instead, periodic records should be continued throughout the experiment, so as to estimate for each dose the time at which one-half of the animals reacted. The estimation of this value from the original data is often facilitated by the use of logarithmic-probability paper or of equivalent techniques, which provide an unbiased estimate of the median time and of the standard deviation without distortion from individuals which react either erratically or not at all (3). The end-point, log-RT50, then represents equivalent levels of susceptibility at all dosages of both standard and unknown and may plot as a straight line against the log-dose. It should then be possible to obtain parallel lines for standard and unknown that are suitable for the estimation of relative potency in terms of *M*. As a criterion for drug assay, the quantitative aspects of reaction time have been explored less fully than those based upon the dosage-effect curve.

(3) Most other biological assays depend upon a graded or quantitative reaction and potency is estimated from the average of the individual responses at one or more dosages of drug. In contrast with an all-or-none reaction, a graded response frequently can be measured in several ways. The reaction to a larger dose may be both greater in intensity and longer in duration than that to a smaller dose, so that a dosage-response curve could be constructed from any one of several terms. Figure 10 shows the course of

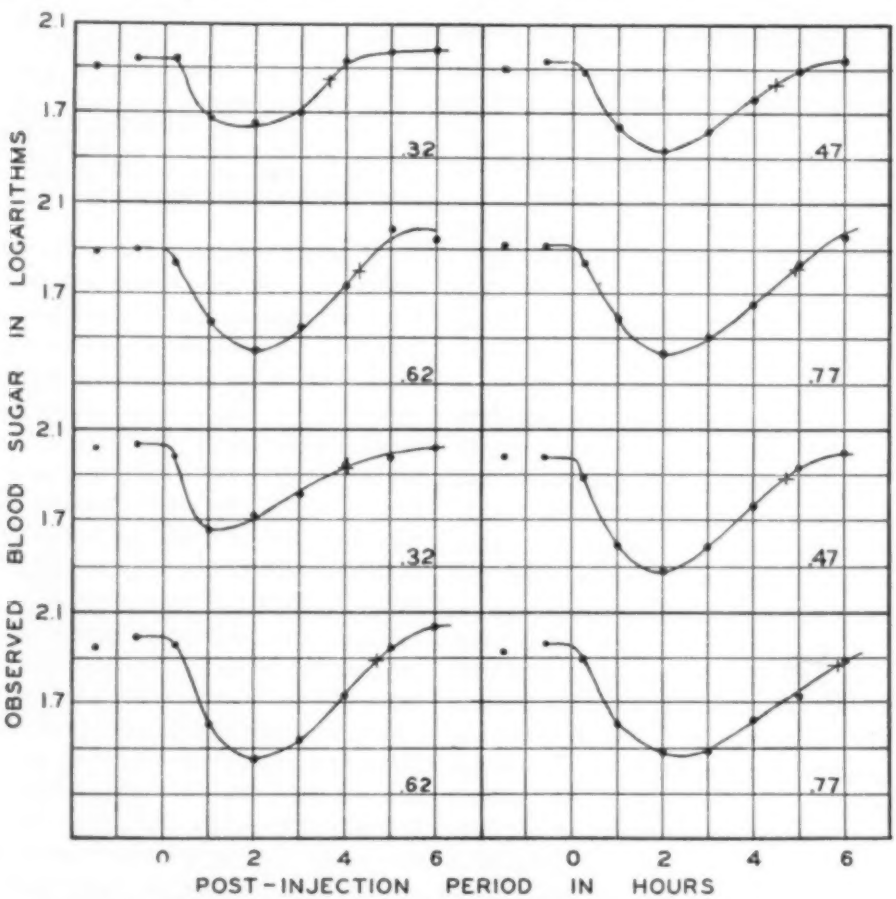

Fig. 10.—Typical Blood-Sugar Curves for an Individual Rabbit Following the Injection of Different Doses (in Logarithms) of Insulin, the Upper Four after One Day without Food and the Lower Four One Day Later, Data of Bliss and Marks (5).

the hypoglycæmic reaction of a typical rabbit to four doses of insulin after one and two days of fasting. The response could be measured from the maximum fall in blood sugar, from the time for the blood sugar to return to a given percentage of the initial value or from the average fall in blood sugar recorded periodically after treatment, and all of these are subject to differences in definition and in their relation to the initial level of blood sugar. Even after eliminating impractical criteria, several alternatives often remain from which one can be selected which will plot as a straight line against the log-dose over a wide range of dosages and have the largest ratio between the slope and the standard deviation about the computed line.

Most dosage-response curves flatten out toward a maximal reaction as the dose is increased or toward a minimum as the dose is reduced, but there is often a long central portion which can be considered as a straight line. In a study of the dosage-response curve for insulin by Bliss and Marks (5), for example, the mean percentage fall in blood sugar based upon five hourly readings was linear over a three- to four-fold change in dose (Fig. 11). In other cases a curvature has been observed at one or both ends and unless it can be rectified by a convenient mathematical transformation, only the central part of the complete curve is suitable for purposes of biological assay. This restriction in the range of response often increases the desirability of including three dosage levels of both standard and unknown in each assay, spaced equally on a logarithmic scale. In other cases, where curvature is exceptional and the use of three doses would prolong the assay undesirably, two doses of standard and two doses of unknown are indicated. In either case a relatively simple method of factorial analysis can be used for computing the log-ratio of the potency of the unknown to the standard (M), the experimental error of the determination (s_M) and a numerical test of the underlying assumption that the dosage-response curves for standard and unknown are equivalent to parallel, straight lines.

The method can be illustrated from a separate computation of the first day's reactions for 8 of the 12 rabbits used in Fig. 11. These data have already been analyzed initially in Table III and we will now examine the effect of dosage as a test assay, on the assumption

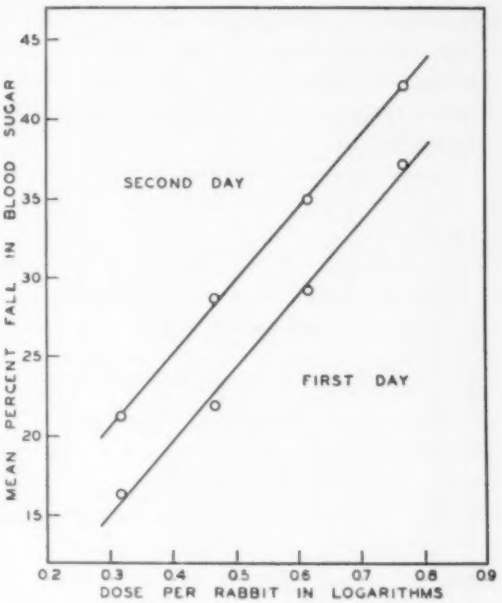

Fig. 11.—Dosage Response Curves Showing the Effect of Insulin upon the Blood Sugar of the Rabbit as Determined from 12 Rabbits, Each Tested Once with Every Dose on Both the First and Second Days of Starvation, from Bliss and Marks (5).

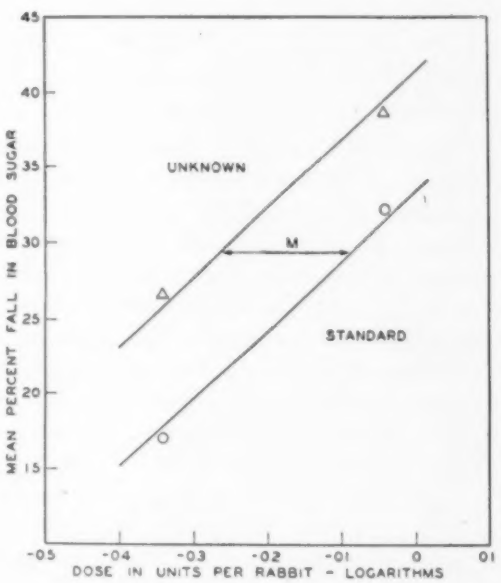

Fig. 12.—A Test Assay for Insulin Where the "Unknown" is a Known Dilution of the Standard, from Bliss and Marks (5).

that doses 1 and 3 represent the standard and doses 2 and 4 an unknown as plotted in Fig. 12. The estimated log-ratio of potencies was $M = 0.179 \pm 0.049$, which differed less than its standard error from the true value of $M = 0.1505$. Since the adjusted $[Y^2]$ measuring the difference in slope between the standard and the "unknown" of Fig. 12 fell within the experimental error, it verified the basic assumption of parallelism.

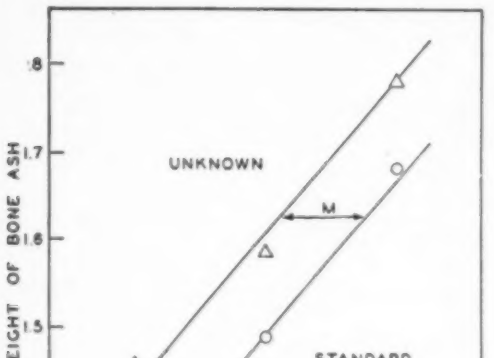

Fig. 13.—The Assay of the Vitamin D Content of a Sample of Cod Liver Oil from the Ash Content of the Bone, Data from Coward (10) as Re-analyzed by Bliss (4).

Bioassays include many unavoidable sources of variation known to the pharmacologist, such as differences between individuals, litters, sexes and dates of treatment. Sometimes these potential sources of error are balanced between the different doses of standard and unknown so that they are as comparable as possible, notably in the so-called cross-over test. This principle has been generalized and improved by means of restricted randomized designs coupled with computation by the analysis of variance, which has been illustrated in Table III. The newer technique not only segregates potential sources of variation, so that the comparisons of doses or of standard and unknown are unbiassed, but it eliminates them from the estimate of experimental error as well. These possibilities for increasing the precision of bioassays are only beginning to be explored.

Assay technique frequently adjusts each individual response for differences in the initial level from which the response is measured, for differences in body weight or for other concomitant factors. These adjustments are often arbitrary and differ from one worker to the next. Necessarily each assumes certain quantitative biological relations between the reaction and the initial or concomitant measure and if incorrect, the adjustment may even reduce the precision of the assay. For example, the use of the percentage ash in the femur of the rat to measure the potency of vitamin D implies a specific relation in the bone between its ash and organic content. This was sufficiently inaccurate in a case reported by Coward (10) and reëxamined by Bliss (4) that it increased the error of M to the same extent as if the number of test animals were halved in comparison with a computation of potency from the log-weight of ash alone without reference to the organic matter lost in ashing. The mean responses to the three doses of standard and of unknown have been plotted in Fig. 13 and in terms of the more accurate criterion $M = 0.199 \pm 0.023$ or upon adjustment for the assumed potency 197.7 ± 10.6 units of vitamin D per Gm. of cod liver oil as compared with 193.4 ± 15.0 units by the original criterion. If adjustment is needed, such concomitant variations can be corrected effectively by the analysis of covariance from the internal evidence of each assay. Covariance has the added advantage of allowing a more flexible assay procedure where several factors are involved as in the official assay for vitamin A.

SUMMARY

The potency of physiologically active agents which are too complex for chemical analysis is determined from the reaction of living matter by biological assay. Five factors characterize good assay procedure: (*a*) substantial equivalence between the activity of the drug in a laboratory test animal and in its therapeutic applications,

(*b*) high sensitivity to a change in dose, measured objectively by the curve relating-dosage and the function of response which over the widest range plots against it as a straight line, (*c*) the definition of potency in terms of a reference standard based upon the reaction to two or more doses of both standard and unknown in each assay, (*d*) the partition of potential variation equally between different dosages and between standard and unknown and (*e*) the determination of its experimental error as an integral part of each assay. Statistical procedures depend in part upon the nature of the response. Those based upon an all-or-none reaction show a sigmoid dosage-effect curve mirroring the individual variation in susceptibility. Since this follows the normal distribution, the curve can be rectified and used effectively for biological assay by transforming dosages to logarithms and percentage effect to probits or their equivalent. Similar procedures are of value when reaction time is used as an assay criterion, providing a biologically stable end-point for each dose. The dosage-response curve for graded reactions is often linear over a manageable dosage range. Especially in these cases the newer statistical designs and methods of analysis extend considerably the precision that can be attained from a given amount of biological material and time.

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Influence of Sex Life upon Resistance to Nostal and Pentobarbital*

By Harald G. O. Holck and Lewis D. Fink†

Agduhr and his collaborators have reported that sex life increases resistance in mice, rats and rabbits to such varied substances as methyl alcohol (1), ethyl alcohol (2), soluble barbital (3) and arsenic trioxide (4). Small, very gradually increasing daily doses were administered until the animals died. Mated female mice showed an increased resistance even though they failed to become pregnant (2). In general, mated female mice gained more in resistance than did the corresponding males (1, 2, 3). However, in the case of arsenic trioxide, mated male rats showed greater resistance. In all of these experiments the animals were divided into three groups: 1, two males together; 2, two females together; 3, male and female together.

EXPERIMENTAL

PART A. OBSERVATIONS WITH A SINGLE DOSE OF ISOPROPYL BROMALLYL BARBITURIC ACID (NOSTAL)

In this phase of the study 81 male and 120 female albino rats, bred from Wistar stock, were divided

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into suitable groups so as to study the effects of living with a member of the same sex (Groups I–IV), sufficient sex life for one male to impregnate one female (V), sex life *ad libitum* for the males (IX), delivery of 1 litter (X), delivery of 2 litters (XI) and partial hysterectomy (VIII, at eight to nine weeks under ether anesthesia), in case of which the finding of semen in the vagina by the method of Frank (5) with at least a two-week interval proved persistence of libido as expected (6) and in which an examination showed presence of normal ovaries at the end of the experiment. In all cases at least twenty days had elapsed between the last delivery and Nostal administration. As far as possible, litter mates were distributed among the various groups. The diet consisted of Purina Dog Chow supplemented by cod liver oil. The rats were kept in air-conditioned quarters at approximately twenty-five degrees Centigrade. The final experiments were conducted on three separate days and each rat was kept in an individual container and well covered; the temperature was twenty-seven degrees C.; a rat was considered recovered when it would promptly retract either hind foot from an extended position. Inasmuch as no consistent definite trend could be found on any of the three days, the results have been combined into one table.

Table I.—Summary of the Results from the Intraperitoneal Administration of 50 mg./Kg. of Nostal (Given as the Sodium Salt) to $4^1/_2$ to $7^1/_2$ Months Old Albino Rats which Had Been Raised under Various Conditions of Sex Life. ϵ = Standard Deviation of the Mean

Group	No. of Rats	Average Hours for Recovery	ϵ of Recovery Time	Percentage of Delayed Death	Combination of Groups	Average Hours for Recovery
		Males				
I—Single males	14	3.4	±0.2	86	I and III	3.1
III—Two males	27	3.0	0.1	81		
IX—Polygamous males	12	3.2	0.2	83	IX and VII	3.3
V—*Male* and F.	15	3.2	0.1	80		
VII—*Males* with F. of Group VIII	13	3.5	0.2	100		
		Females				
II—Single females	15	3.7	±0.2	100	II and IV	3.6
IV—Two females	29	3.6	0.1	100		
VIII—Females with uteri excision	13	4.0	0.2	100		
X—1 Delivery F. with VI *Female* and M.	36	3.4	0.1	86	VI, X and XI	3.3
XI—2 Delivery females	27	3.3	0.1	89		

The differences in recovery time and percentages of delayed death are all within the limits of normal variations for this dose of Nostal (7). Hence there is no indication that any of the experimental conditions of sex life have an effect upon either of these criteria. A closer examination of the complete data shows that even when the time between delivery and injection was only three to six weeks, there still was no certain benefit. Yet, it is possible to influence the resistance to Nostal, as can be seen from the following data. Six doses of an average of 10 mg./Kg. of Nostal were administered at three- to four-day intervals to 18 hybrid rats (10 males, 8 females); four days after the last of these small doses, the regular dose of 50 mg./Kg. of Nostal was given; none died delayed death; of 17 non-treated rats (8 males, 9 females), 83 per cent died within a few days following the 50 mg./Kg.

In view of the fact that Agduhr has reported an increase in the secretory mass of the thyroid gland (8) and Carlson *et al.* (9) have found rearrangement of the adrenal cortex during pregnancy with increased lipoid and diminished vitamin C contents, we decided to fix the thyroid, adrenal and pituitary glands thoroughly in 4 per cent formaldehyde solution, to weigh them and to compare their relation to body-weight, although the body-weights had decreased to a variably marked degree when the rats had died from pulmonary complications following Nostal administration or had been killed on the fifth day (7). Within the groups of either sex, no significant differences could be detected in the average percentages of the weights of any of these glands; however, the proportion of the female thyroid to body-weight was slightly greater, that of the pituitary about twice and that of the adrenal about three times as great as in the corresponding glands of the male. A check upon five male and five female controls (killed acutely with chloroform) gave approximately the same sex-difference, using the weights of either the fresh or the fixed glands. These last two sex-differences in our stock were distinctly greater than have previously been reported by Donaldson (10) and Hatai (11).

PART B. OBSERVATIONS WITH GRADUALLY INCREASING DOSES OF SODIUM PENTOBARBITAL (ETHYL 1-METHYL-BUTYL BARBITURIC ACID; NEMBUTAL) GIVEN EVERY 90 MINUTES

In these experiments we used 74 male and 103 female albino rats. The mated pairs (Group III) and pairs of the same sex (II, VIII) were kept together constantly as in Agduhr's tests. To study the effect of coexistence of the male and female without sexual intercourse, a small wound clip was carefully fastened under ether anesthesia around the vaginal tract just under the surface of the skin

through a lengthwise incision a centimeter and a half to the side of the vaginal opening, with subsequent closing of the incision. Although in 13 of the 18 females no clip was found at the time of barbiturate injection, adhesions had closed the vaginal opening and none of the females in this group (VII) with the vaginal "chastity" clips became pregnant, although caged for about two months with males, each of which had (*a*) impregnated one female (Group V) and (*b*) had had repeated coition with a female with partial uteri excision (VI).

that part of the time they ate and apparently became normal between injections until later on they again became depressed and ultimately died. In a few cases the rats were found to be normal before each subsequent injection for 60 or more periods. Development of tolerance to Pentobarbital in the rat is in agreement with the findings of Moir (12) and Stanton (13), who gave this drug in repeated small doses over a period of several weeks.

The results of these tests have been summarized in Table II. They show that sex life had no influence upon the male resistance, that living with the male without copulation, or sexual intercourse without pregnancy did not influence the female resistance, but that this was raised distinctly in females that were or recently had been pregnant, although the resistance was not brought up to that of the males.

Table II.—Summary of Results from Albino Rats, 5 to 6 Months Old, Injected Subcutaneously Every 90 Minutes with Gradually Increasing Doses of Nembutal until Death

Group	No. of Rats	Average Total Dose (mg./Kg.)	Percentage of Rats Developing Tolerance	Chi Square Method Compared with Group	Factor	Remarks
				Males		
I—Males with F. of V, VI and VII	22	1372	77	All 74 M. with the 39 "pregnant" females	20	"Pregnant" = were or had been pregnant
II—Two males	31	1475	77			
III—Male and F.	21	1465	81			
				Females		
IV—Female and M.	21	771	30	All 64 "non-pregnant" F. with the 39 "pregnant" females	17	IV 38% pregnant and 43% lactating at time of injection
V—1 delivery F.	18	716	44			
VI—Females with uteri excision	23	202	4			V 6% pregnant and 94% lactating at time of injection
VII—Clip vagina females	18	235	6			
VIII—Two females	23	198	4			

We administered doses of Sodium Pentobarbital which were very slowly increased logarithmically. The first dose was 12.16 mg./Kg.; this was increased by 5 per cent for two more doses, and from then on by 2.24 per cent. The highest dose given was 94.3 mg./Kg., or 7.7 times the initial dose. Twelve sites of injection under the skin were used in rotation. The 1 per cent solution was freshly prepared 33 times during the experiment which lasted eight days and nights. The sample of Nembutal used was lot 75952, concerning which it was known that delayed death did not occur and to which our male rats showed a distinctly higher resistance than the females. General care of the rats was the same as in Part A, and the final test again was conducted at twenty-seven degrees C. At the end of every 90 minutes the condition of each rat was recorded, except when it had died between injections.

By this technique it was possible: 1, to observe constantly each experimental rat; 2, to record more exactly the time of death; 3, to notice whether tolerance was developed during the experiment; 4, to avoid the possible killing by a cage mate of a rat enfeebled by the drug. Two main types of responses occurred. In one type the rat became deeply depressed after a few doses of Pentobarbital, thence continued to lie on the side and died without having shown any visible signs of improvement. In the second type of response the rats in the beginning became depressed and lay on the side, but after a varying number of injections improved so

PART C. FURTHER STUDIES UPON THE EFFECTS OF GRADUALLY INCREASING DOSES OF SODIUM PENTOBARBITAL GIVEN EVERY 90 MINUTES

The main object of this experiment was to establish more firmly that sex life has no influence upon male rat resistance (Groups I, II), but that pregnancy and recent pregnancy (III, IV, V) notably increase the resistance to Pentobarbital. A group of 10 young virgin females (VI) was added to make possible a comparison with the older females; in case of 6 of these young rats which had received more than fifty doses of Pentobarbital and had shown visible tolerance, the administration was discontinued to see if permanent recovery would occur. Another group of 13 rats (VII) was included to observe if tolerance produced by preliminary injections of suitable doses of Sodium Pentobarbital over several weeks (as was previously done with Nostal) would raise the resistance appreciably. Finally, one control rat was given 60 doses of one and another rat the same number of doses of one and one-half times the amount of saline used as a solvent for the barbiturate used in these tests.

The care of the rats and general technique were the same as in the previous experiments, except that we started with a smaller dose, 11.7 mg./Kg., that the increases were uniformly 3.25 per cent, which somewhat shortened the experiment, that only 10 sites of subcutaneous injection were used in rotation, and that we used another sample of Pentobarbital (Lot No. 3032X947503), of which we also knew that sex-difference existed in our stock.

The main results are summarized in Chart 1. Again, there was no significant difference between mated males and males kept in pairs. On the other hand, as before, pregnant or recently pregnant females developed tolerance about ten times more often than did the virgins and the average total fatal dose was five times greater in the former and approached male fatal dosage more closely than in Part B. However, young virgins also developed tolerance about ten times more frequently than did older virgin females; hence, age is an important factor in this type of study. This is of interest in connection with Agduhr's observation (2) that resistance of mice to alcohol seems to be the greatest during the period of most rapid growth; also in connection with our own studies upon Evipal (15), in which the female rat response to single doses gradually increased between the ages of one and four months; however, the opposite occurred in the males. We are unable to state whether the average fatal dose of Sodium Pentobarbital for the young virgins would have exceeded that for the older males, because we ceased injections in the case of 6 of these females which had developed tolerance to see whether they would recover permanently from doses varying from 1572 to 1653 mg./Kg. Uneventful recoveries took place and these rats apparently were in good health and growing two weeks later. Likewise, an older control female recovered readily from 60 doses of the saline solution used as a solvent for the barbiturate, and another one from 60 doses of saline solution one and one-half times that large. The preliminary administration of small doses of Pentobarbital at intervals of several days for several weeks prior to the main experiment, the technique so eminently successful in preventing delayed death when Nostal was used, failed to raise the average resistance of virgin females to a level near that of rats that were or recently had been pregnant, although there was a measurable increase in the average fatal dose over that for virgin rats

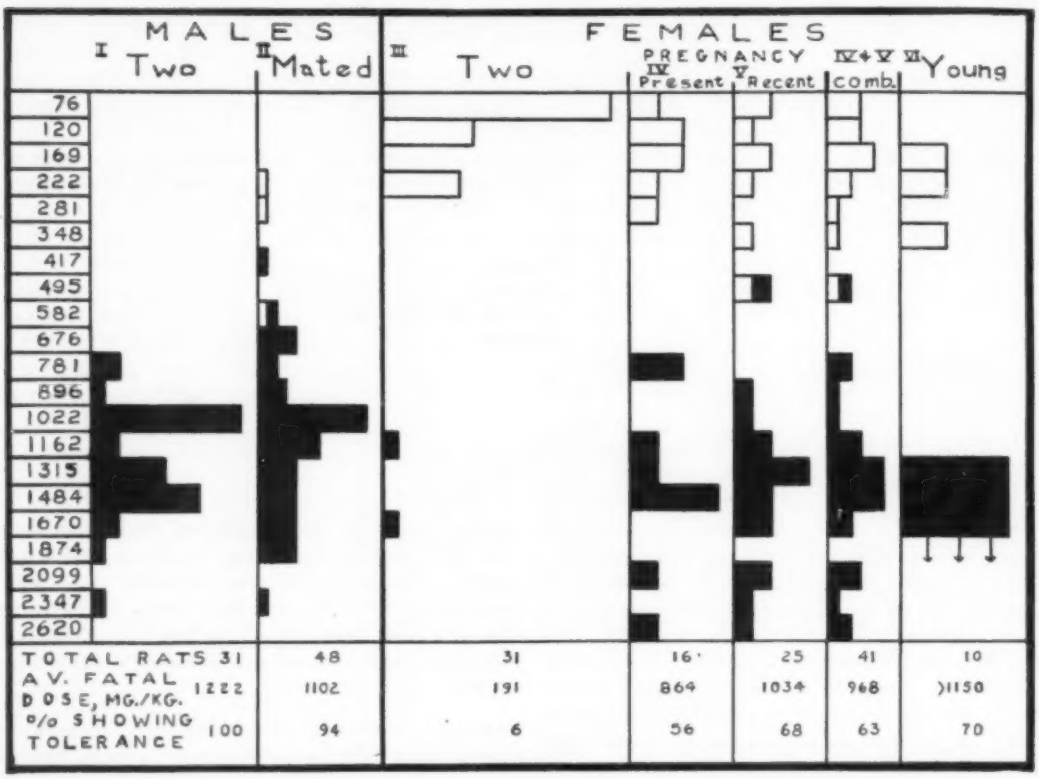

Chart 1.—Percentage Mortality Distribution and Tolerance (in Black) of Various Groups of Albino Rats Injected Subcutaneously with Gradually Increasing Doses of Sodium Pentobarbital. Successive Three Doses Are Combined. Because of Absence of Mortalities on the First Three Doses, They Are Omitted. The Rats Were Five to Seven Months Old Except the Young Which Were Two to Two and One Half Months Old. Recently Pregnant Means up to 39 Days after the Last Delivery. Downward Arrows and > Show That the Total Fatal Dose Is Higher Than Indicated, Due to Discontinuation of Injections to See Whether These Rats Would Permanently Recover.

which had received no treatment. However, it must be borne in mind that the result of a single rat that develops tolerance exerts a much greater influence upon the average when the group is small (only 13 rats constituted this group; 1 developed tolerance and the average total fatal dose was 279 mg./Kg.).

DISCUSSION

The outstanding result of our experiments with gradually increasing doses of Sodium Pentobarbital (Nembutal) is that among the varying conditions of sex life in both sexes of the albino rat, only pregnancy markedly increased the incidence of tolerance and, consequently, the average total fatal dose, and that this effect persists at least during the period of lactation. Just how permanent this effect is will be settled by future tests. It should be noted that our technique of Pentobarbital or of Evipal (methyl cyclo-hexenyl *N*-methyl barbiturate) administration exaggerates any existing sex-difference so that by proper adjustment one may kill nearly all of the females selectively (14, 15). In past experiments with this Pentobarbital sample, the male L.D. 50 was only about sixty per cent higher than that of the female when single doses were administered to groups of our stock, whereas in the present study it was about seven times as great. Hence, to be certain of a significant difference by the technique of daily administrations as carried out by Agduhr or by our 90 minute method, one must secure much greater differences in the average total fatal dose than by determining the average single fatal dose. Therefore, we have stressed the large increase in animals showing visible tolerance, rather than attempting to interpret statistically the combined results of rats showing tolerance and those not showing tolerance in the same group.

Although the literature reports internal changes in the thyroid, adrenal and pituitary glands as a consequence of pregnancy, we were unable to find that the varying conditions of sex life in our study modified the average proportion of the weights of these glands to body-weight; however, there was a greater sex-difference in case of the adrenal and pituitary glands in our stock than has been previously reported by several authorities. A further analysis would seem to necessitate studies upon the various hormones, the amounts of which are changed during pregnancy. It is of interest in this connection that an anti-narcotic action has been reported for both androgens and estrogens (15, 16).

It should also be noted that young virgin female rats showed a resistance at least similar to that of pregnant or recently pregnant older female rats. That such a relation may hold for all drugs seems *a priori* improbable because young rats do not show higher resistance to all drugs, but to some an equal and to others a distinctly lower resistance than the adult; and, as mentioned, even with the same substance there may be a difference in this respect in the two sexes (15). In case of nicotine it has been reported that tolerance to small doses was developed by growing rats only (17). A summary of the effect of age in the rat has recently been prepared (18). The same caution holds in regard to drawing too far-reaching conclusions from the effect of pregnancy in this case. It is true that pregnant rats tolerated doses of thyroid and thyroxin which in non-pregnant rats caused rapid loss of weight and death (19). But, on the other hand, pregnant rats have been reported especially sensitive to combined administration of tyramine and phenylethyl amine (20). It is also an open question what effects pregnancy would have upon female rats in those cases in which the female rat has a higher resistance than the male, as in case of morphine (21), ouabain (22) or even the amino acid, tyrosine (23).

SUMMARY

1. Various conditions of sex life did not significantly influence male or female albino rat recovery time or percentage of delayed deaths from 50 mg./Kg. of Nostal (isopropyl bromallyl barbituric acid), administered intraperitoneally.

2. No difference in resistance to gradually increasing subcutaneous doses of Sodium Pentobarbital (Nembutal; ethyl 1-methylbutyl barbituric acid) could be demonstrated in male rats raised under varying

conditions of opportunity for copulation, or in females caged with males with sexual intercourse established but pregnancy prevented through partial hysterectomy, or in normal females coexisting with males but with coition prevented by a vaginal "chastity" clip.

3. The resistance to such medication was markedly raised in females which were or recently had been pregnant, but was not brought up to that of the males.

4. Young virgin females developed tolerance to gradually increasing doses of Sodium Pentobarbital about ten times more often than did older virgins. Six young rats recovered permanently from medication every 90 minutes for three days and nights.

5. Small doses of Nostal administered at three- to four-day intervals for several weeks completely prevented delayed deaths from 50 mg./Kg. of Nostal, which killed 83 per cent of the controls.

6. Similar preliminary treatment with Sodium Pentobarbital raised the average resistance of virgin female rats only moderately.

7. Varying conditions of sex life did not alter the average percentage weights of the thyroid, adrenal or pituitary glands. The higher proportion of the latter two glands in the females was greater than usually has been reported for the albino rat.

We are indebted to Miss Lucille M. Mills and to Mr. Donald R. Mathieson for aid in this work.

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A Phytochemical and Histological Study of *Purshia tridentata* (Pursh) D.C.*

By Charles V. Netz, Charles H. Rogers and Glenn L. Jenkins

Purshia tridentata (Pursh) D.C., family Rosaceæ, is a diffusely branched shrub grow-

* Presented to the Scientific Section, A. PH. A., Richmond meeting, 1940.

Abstract of thesis presented to the Graduate Faculty of the University of Minnesota by Charles V. Netz in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

From the laboratories of the College of Pharmacy, University of Minnesota.

This is a report in a coöperative research project connected with the Indian Medicinal Plant study of the Bureau of Plant Industry, United States Department of Agriculture.

ing to a height of 3–5 feet and bearing clusters of cuneate, 3-lobed, small leaves. The small yellow flowers are not prominent. The fruit is a soft, hairy achene. Synonyms are Antelope Brush and Bitter Brush. The plant grows from the western slope of the Rocky Mountains to the mountains of California and northward.

The plant material (serial number H 496) was collected in the summer of 1938 in Washoe County, Nevada, by James R. Henrichs and was identified by Rogers McVaugh of the Division of Plant Exploration and Introduction, Bureau of Plant Industry of the United States Department of Agriculture, Washington, D. C. It consisted of the much-broken, air-dried, over-ground portion containing very few fruits and flowers.

Decoctions, infusions and poultices of the over-ground portions of this plant have been used for years by the Indians of that region for the treatment of pathological conditions and infections ranging from sore eyes and internal hemorrhage to measles, smallpox and gonorrhea. This investigation was intended to isolate and identify any constituents responsible for the purported curative action obtained by the Indians.

Trimble (1) reported a preliminary investigation of the fruit in which he found a "bitter substance," oily constituents, resins, tannins, mucilages, pectin and phosphates of calcium and magnesium.

EXPERIMENTAL

HISTOLOGICAL

The inner morphology of the stem and leaf of *Purshia tridentata* D.C. was studied by means of transverse and radial- and tangential-longitudinal sections supplemented by a microscopic examination of the elements in the powdered material.

The stem revealed a typical dicotyledonous structure with pith parenchyma filled with tannin, xylem, cambium, phloem and bark, the last containing from one to four layers of cork cells and numerous groups of stone cells. The vessels were arranged diffusely and were characterized by oblique and bordered pits and simple end-wall perforations. Spiral trachea and calcium oxalate rosettes were also present.

The leaf showed epidermis, palisade cells, collenchyma, spiral trachea and calcium oxalate rosettes. The adaxial surface was glabrous whereas the abaxial surface bore two types of uni-cellular,

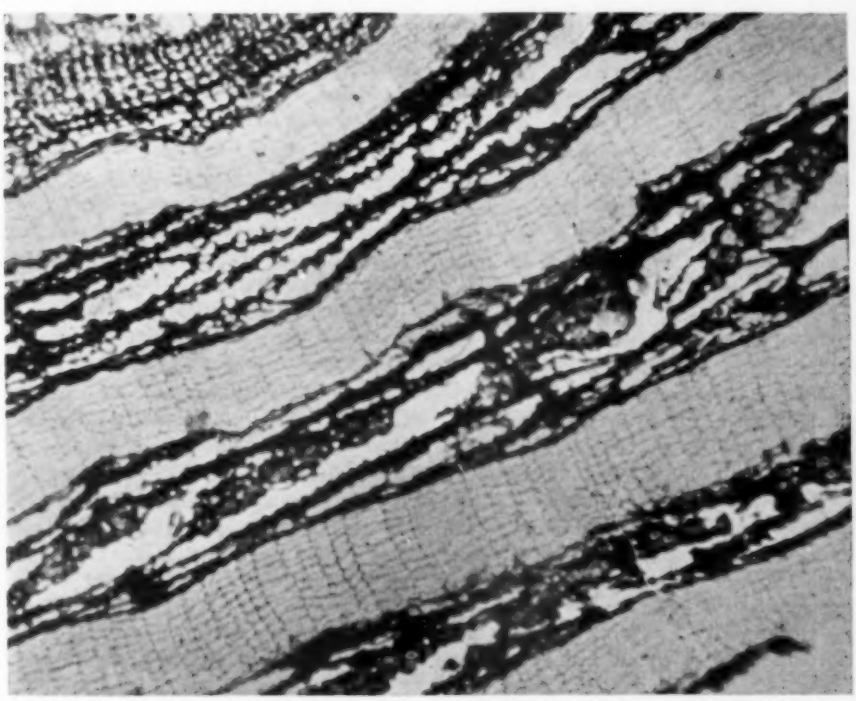

Fig. 1.—Transverse Section of Bark × 100. Stained with Safranin and Haidenhain's Iron-Alum Hæmatoxylin.

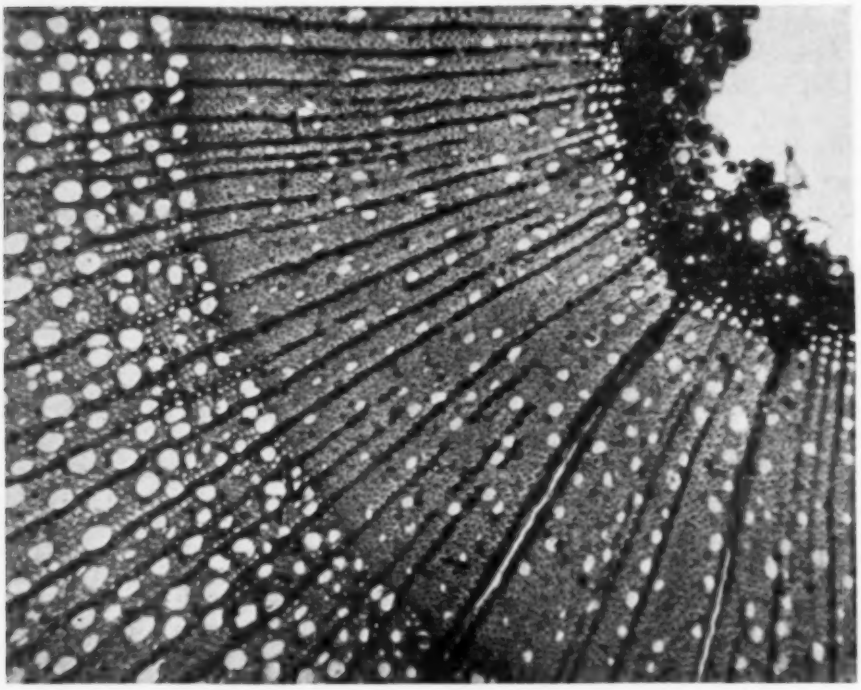

Fig. 2.*—Transverse Section of Wood × 100. Stained with Safranin and Haidenhain's Iron-Alum Hæmatoxylin.

non-glandular trichomes, one short and pointed, the other long and irregularly curled.

PHYTOCHEMICAL

The plant material was reduced to a No. 20 powder and the following constants of the well-mixed powder were determined by U. S. P. methods: total ash 3.12%, acid-insoluble ash 0.39% and moisture 6.91%.

Preliminary Examination.—An infusion of the powdered plant material made with hot water was acid to litmus paper, gave tests for tannins with ferric chloride T.S., and gelatin-salt T.S. and produced a foam indicative of the presence of saponins but gave no test for starch.

Examination of portions of the powder under a microscope showed a few starch grains.

Steam distillation did not yield an appreciable quantity of volatile constituents. Dilution with alcohol of portions of the filtered decoction remaining from the steam distillation produced a stringy, mucilaginous precipitate indicative of mucilage or pectin (2). The filtrate from this precipitate, after being dealcoholized, gave tests (3) which indicated that the tannin present in the plant material was a catechol or catechol-phloroglucinol tannin (Stenhouse-Proctor classification) (4).

Tannin.—A quantitative aqueous extract of the plant material was freed of constituents insoluble in 70% alcohol, and the tannin content was determined by the hide powder method of the A.O.A.C. (5). The tannin content was 6.55%.

Alkaloids, Glycosides and Sucrose.—Extraction of a portion of the powdered plant material with Prollius' fluid and ammonia water and extraction of additional portions with weak hydrochloric acid at 80° and 100° C., respectively, and Prollius' fluid failed to yield alkaloids.

The method of Stas-Otto (6) applied to a portion of the powdered plant material indicated that no glycosides or alkaloids were present.

Tests for cyanogenetic glycosides by methods suggested by Rosenthaler (7), using sodium picrate paper, gave negative results.

The Bourquelot method (8) using yeast and emulsin showed the absence of sucrose but gave indications of the presence of a glycoside.

Protein.—The plant material contained 8.37% of protein as determined by the Kjeldahl method (9).

General Method.—The general method of extraction of the plant material by a series of organic solvents (10) was applied to 50 Gm. The petroleum ether extract contained a plant wax and a small quantity of material tentatively identified as a fixed oil or an aromatic ester. The ether extract contained a resin. The chloroform extract yielded additional resin. The alcohol extract contained tannins, carbohydrates and reducing substances. The ethyl acetate extract yielded a very small quantity of phlobaphenes.

* The original thesis contains 35 photomicrographs of the plant sections.

Petroleum Ether Extract.—About 75 Gm. of the powdered plant material were extracted with petroleum ether in a Soxhlet extractor. The plant material (*A*) was reserved for use in the lead method. The petroleum ether extract contained a wax and a fixed oil or an aromatic ester which upon treatment with alkali and acid yielded an organic acid with a melting point of 38.0° C.

Lead Method.—The lead method (11) for the separation and identification of the hot-water soluble constituents was applied to *A*. The precipitate obtained with lead acetate contained tannins and mucilages or pectins; the precipitate from basic lead acetate contained tannins and either mucilage or pectin or both and gave indications of the presence of glycosides; the filtrate from the lead precipitates gave tests for reducing substances and carbohydrates but no tests for glycosides or alkaloids.

Pectin and Mucilage.—A decoction of the powdered plant material was diluted with alcohol until the concentration of the alcohol in the mixture was 70%. The stringy, mucilaginous, brown precipitate which separated was filtered out and purified. The presence of pectin in this purified material was indicated by jelly formation with sucrose (12) and precipitation from aqueous solution with calcium chloride T.S. (13). The presence of either pectin or mucilage or both was indicated by the presence of methyl alcohol after hydrolysis (13), the presence of calcium and magnesium in the ash (14), precipitates formed in aqueous solution with certain reagents (15) and the production of mucic acid upon oxidation (16).

The powdered plant material contained 0.626% of pectin weighed as calcium pectate (17). The approximate factor 2.041 was developed experimentally to convert calcium pectate to equivalent pectin. When multiplied by 0.626%, it gave 1.28% of pectin in the plant material.

The 70% alcohol filtrate from the precipitation of pectin and mucilage was detannated with magnesium oxide. The filtrate contained reducing substances and carbohydrates which yielded an osazone with a melting point of 195.5° C., a value conforming to that for galactosazone. The filtrate gave no test for pentose sugars (18).

Alcoholic Extract.—About 3 Kg. of the powdered plant material were extracted with alcohol in a Barnstead extractor, the liquid extract was evaporated to dryness and the residue was designated as "Barnstead extract."

A portion of this "Barnstead extract" was extracted with boiling alcohol, followed by boiling water. The alcohol extract contained a wax with a melting point of 69.8° C. and a resin, and gave tests for carbohydrates and reducing substances. The brown aqueous extract gave tests for tannins, carbohydrates and reducing substances. Hydrolysis with hydrochloric acid of aqueous solutions of the residues from the evaporation of both of the liquid extracts produced no change in optical rotation or no increase in copper reducing action when determined by the method of Shaffer-Somogyi (19). This indicated the absence of a glycoside. The portion of the "Barnstead extract" which was insoluble in alcohol and water consisted largely of phlobaphenes, and an ash of a portion gave tests for phosphates, magnesium, calcium, sodium and potassium.

General Method: A portion of the semi-solid "Barnstead extract" was mixed with an equal weight of clean, dry sand and this mixture was subjected to extraction (10) by a series of organic solvents. The petroleum ether extract yielded a plant wax and a semi-solid fatty material which upon saponification and acidification of the alkaline mixture gave an organic acid with a melting point of 41.5° C. The ether extract contained a small quantity of a brittle, brown solid which appeared to be a resin of indefinite melting point. The chloroform extract yielded a small quantity of a solid which appeared to be the same as that obtained from the ether extract. The alcohol extract contained tannins, phlobaphenes and carbohydrates and the ethyl acetate extract left a residue of tannins and phlobaphenes. An aqueous extract yielded a small quantity of tannins and carbohydrates.

Aromatic Ester, Sterol and Resin: The remainder of the "Barnstead extract" was extracted with boiling alcohol and the liquid extract was concentrated and refrigerated to remove wax. This was filtered out, the filtrate was absorbed upon purified oak sawdust and the mixture was oven dried. The dry product was extracted in a Soxhlet with petroleum ether followed by ethyl alcohol.

The petroleum ether extract was evaporated to dryness. The semi-solid, green-black residue (*O*) had an acid value of 50.86, a saponification value of 151.26 and an ester value (calculated) of 100.40. An ether solution of a portion of the residue was extracted with 1% ammonium carbonate (*B*), 1% sodium carbonate (*C*), 0.1% sodium hydroxide (*D*) and 1% sodium hydroxide (*E*) solutions (20). The ether solution which remained was labeled *M*. Acidification of the alkaline extracts yielded an organic acid in each case. The acids from *B*, *D* and *E* were too small in quantity to permit of a melting point determination. The acid from *C* had a melting point of 51.0° C.

Ether solution *M* was evaporated to dryness, the dark green residue was refluxed with potassium hydroxide solution and the alkaline solution was extracted with ether. The residue from this ether extract gave no tests for sterols or for alcohols although an alpha-naphthyl urethane derivative (m. p. 296.8° C.) was obtained. The alkaline solution was next acidified and extracted with ether. This ether extract contained an organic acid with a melting point of 42.4° C.

Another portion of the petroleum ether residue (*O*) was distilled in a Hickmann vacuum still. The distillate contained a wax, a sterol and a brownish black ester which yielded an organic acid. The oily, pale yellow distillate had an acid value of

49.47, an ester value of 113.1 and a saponification value (calculated) of 162.6. The ether extract of a saponified mixture yielded a sterol and a yellow, semi-solid alcohol which had an odor resembling nutmeg. An ether extract of this saponified mixture after it had been acidified, contained an organic acid with a neutralization equivalent of 297.6. The acid formed a *p*-bromophenacyl bromide ester with a melting point of 69.0° C. The aqueous-acid solution after extraction with ether did not contain glycerin, indicating that the distillate did not contain a glyceryl ester (21).

The dark green alcohol extract from the sawdust mixture was concentrated in volume and diluted with 5 volumes of ether to precipitate tannins, sugars, etc. The precipitate was filtered out and the filtrate, which contained resins, was evaporated to dryness. The residue, considered to be resin, had an acid value (quinhydrone electrode) of 2.17, a saponification value (glass electrode) of 235.8 and an ester value (calculated) of 233.6.

An ether solution of this residue was extracted with 1% ammonium hydroxide (*F*), 1% sodium carbonate (*G*), 0.1% sodium hydroxide (*H*) and 1% sodium hydroxide (*I*) solutions. The ether solution which remained was labeled *N*. Acidification of the alkaline extracts yielded organic acids, the quantity from *I* being too small for tests. The acid from *E* had a melting point of 137–144° C., that from *G* a melting point of 186.6° C. and formed an ethyl ester with a melting point of 152.4° C. and that from *H* a melting point of 196–201° C. and formed a *p*-bromophenacyl bromide ester with a melting point of 151–171° C. Ether solution *N* was evaporated and the residue was saponified. Ether removed from this saponified mixture a small quantity of a substance which would not give a test for the hydroxyl group or would not form a derivative. Extraction with ether of this saponified mixture after it had been acidified, yielded an organic acid (m. p. 54.7° C.) which would not form the usual derivatives.

PHARMACOLOGICAL*

A fluidextract prepared by Type Process A of the U. S. P. produced a lowering in the blood pressure of a rabbit. When administered orally with the ration, it had no beneficial effect upon Pneumococcus Type II infections in mice. It had a bacteriostatic effect upon 4 of a series of 12 microörganisms *in vitro*. That this action resided in the tannin was indicated by the fact that a detannated fluidextract had no bacteriostatic effect upon any of the 12 microörganisms.

*The pharmacological and bacteriological tests were conducted by Dr. Raymond Bieter, Dr. Winfred P. Larson, Milton Levine, Elizabeth M. Cranston and Charles Drake of the School of Medicine, University of Minnesota.

SUMMARY

The histological investigation showed a structural arrangement typical of the dicotyledons with secondary growth. The abaxial surfaces of the leaves had two types of uni-cellular, non-glandular hairs.

The phytochemical investigation showed the presence of a catechol or catechol-phloroglucin tannin, a bitter substance, starch, pectin, mucilage, sterols, an aromatic ester, a wax, an ester-resin, small quantities of resin-acids and galactose. No alkaloids or glycosides were found. The total and acid-insoluble ash, moisture and some of the constants of the ester and resin were determined as well as the quantity of protein and tannin and an approximation of the quantity of pectin and mucilage.

CONCLUSION

Purported curative action obtained by the Indians from infusions, decoctions and poultices of this plant was probably due to the bacteriostatic action of the tannins and the demulcent effect of the mucilage and pectin.

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A Comparative Study of the Total Volatile Acids of Viburnum Stem and Root Barks*

By Irvine W. Grote and Charles Colburn†

Two species of Viburnum are official in N. F. VI, namely, *V. prunifolium* and *V. opulus*. The latter is a small shrub and since its introduction into U. S. P. VII the "dried bark" has been official. *V. prunifolium* was official in U. S. P. VI and VII as the "dried bark." In U. S. P. VIII, the root bark was specified, but this was changed back to the "dried bark" in U. S. P. IX and again changed to "root bark" in N. F. V and VI. In U. S. P. VIII and IX, *V. lentago* could be used as *V. prunifolium*. Examination of the literature does not reveal any scientific reason for this alternate change from *V. prunifolium* whole bark to root bark. Continued collection of the root bark of *V. prunifolium* will lead to a growing scarcity, while, if merely the stem bark is collected, the tree is quickly replaced by shoots from the original root system. This paper represents the first in a series of comparisons by chemical means of the stem and root barks of *V. prunifolium*.

Both Valerian and Viburnum are peculiar in that they are reported to contain large amounts of valeric acid compounds as well as unusually large amounts of other volatile aliphatic acids released upon hydrolysis. We have found in our own laboratory that the bark of the various Viburnums release several times as much volatile acid following hydrolysis as do such barks as *Cascara Sagrada*, etc. Many workers have assumed that the valeric acid compounds are responsible for the uterine sedative action of the two drugs. Heyl and Barkenbus (1) made perhaps the most careful study of *V. prunifolium* root bark and report after hydrolysis the presence of the volatile acids formic, acetic, valeric and traces of butyric.

No simple direct methods for accurate quantitative determinations of the separate aliphatic acids in presence of several others of the same series could be found in the literature. The steam distillation method of D. C. Dyer (2), however, seems to afford a useful and rapid method of comparison of the amount of one mixture of volatile acids with another and to determine any marked variation in composition of the acids in the mixture. The method consists of steam distillation of the acids from a constant volume of solution and titration of successive 100-cc. fractions of the distillate with alkali. Each of the lower aliphatic acids steam distils at a constant rate proportional to the amount of volatile acid still present in the constant volume. The rate for the acids, through caproic at least, is characteristic for the individual acid and is the reverse of what would be expected, caproic acid distilling at the most rapid rate and formic acid at the slowest rate. Mixtures of the acids distil at rates proportional to the amount of each acid present in the mixture.

In view of the apparent importance of the volatile acids in Viburnum bark, and their possible relation to physiological action, it seemed worth while to apply Dyer's method in a comparison of stem and root barks of *Viburnum prunifolium*. The method was modified in that no attempt was made to keep the volume of liquid constant but rather to run the distillations under identical conditions. In addition, Dyer has shown that over twenty successive 100-cc. fractions were required to remove substantially all the formic acid. This would make the method unnecessarily long for comparative purposes. By the fifth successive 100-cc.

* Presented before the Scientific Section, A. PH. A., Richmond meeting, 1940.
† From the Department of Chemistry, University of Chattanooga, Chattanooga, Tennessee.

fraction all the acids down to propionic are removed, as well as two-thirds of the acetic and one-half of the formic. Since we are largely interested in the higher volatile acids, and then only in comparing one drug with another under like conditions, the distillation was carried through only five 100-cc. portions in one series and through six 100-cc. portions in another. In this latter series, larger flasks and a slightly different technique were used. Both methods gave results differing in the figures obtained but strictly comparable as to results. Only one series is reported in this paper. The procedure used was as follows:

EXPERIMENTAL

Twenty grams of the bark ground to 5–10 mesh are hydrolyzed on a water bath with 100 cc. 5% sulfuric or phosphoric acid in a 275 sulfur flask fitted with a reflex condenser. The same flask, while still hot, is then fitted with a steam inlet tube reaching to the bottom and an outlet tube passing through a Kjeldahl bulb to a condenser. Steam generated in a 1 l. boiling flask is passed in at such a rate that the distillate is collected at about 5 cc. per minute. By arranging the sulfur flask so as to touch the boiling flask, five 100-cc. portions can be conducted over without the flask overflowing. Five successive 100-cc. portions are collected in short 100-cc. cylinders and titrated in a cumulative manner by adding each 100-cc. portions to a 750-cc. flask, using half-normal sodium hydroxide and phenolphthalein indicator. As reference standards, 1 cc. of the aliphatic acids from formic through caproic (Eastman) were added to twenty grams of acid exhausted Viburnum bark and 100 cc. 5% sulfuric acid and the mixture heated for six hours on the water bath and then steam distilled. The results obtained are given in Table I. Mixtures of the acids show changing rates of distillation varying distinctly from those of the individual acids, since the higher acids are more quickly removed. In order to show easily comparable figures, the titration figures in cubic centimeters were also calculated as to percentage distilled in each fraction by the formula

$$\% = \frac{\text{total cc. of } N/2 \text{ NaOH used through this 100-cc. fraction } x\ 100}{\text{total cc. of } N/2 \text{ NaOH used in titration of the five 100-cc. fractions}}$$

Table I.—Steam Distillation Rates of Approximately 1-Cc. Portions of the Lower Aliphatic Acids Mixed with 20 Grams Exhausted Viburnum Bark and 100 Cc. 5% Sulfuric Acid

Acid	Cc. $N/2$ Alkali Required per 100-Cc. Portion						Cumulative % of Total				
	1	2	3	4	5	Total	1	2	3	4	5
Formic	7.2	5.4	4.0	2.3	2.1	21.0	34	60	79	90	100
Acetic	10.9	6.5	3.1	1.8	1.8	24.1	45	72	85	92	100
Propionic	14.3	5.3	2.6	1.0	0.2	23.4	61	83	95	99	100
Technical butyric	13.0	3.2	1.4	0.8	0.2	18.6	70	87	94	99	100
N Butyric	15.8	4.5	1.7	0.8	0.5	23.3	68	90	94	98	100
Isobutyric No. 2	16.0	2.6	0.6	0.2	0.0	19.4	82	96	99	100	...
N Valeric	17.0	2.5	0.6	0.1	0.0	20.2	84	91	99	100	...
Isovaleric	14.4	1.7	0.3	0.0	0.0	16.4	87	98	100	...	...
N Caproic	13.6	1.3	0.3	0.0	0.0	15.2	90	98	100	...	...
Isocaproic	12.5	1.1	0.3	0.0	0.0	13.9	89	97	100	...	...

Table II.—Commercial Root Bark Samples

(Each figure represents an average of two or more determinations on 20 Gm. representative samples.)

Drug	Cc. $N/2$ Alkali per 100-Cc. Frac. Dis.						Cumulative % of Total				
	1	2	3	4	5	Total	1	2	3	4	5
Prunifolium No. 1	11.5	3.5	2.6	2.1	1.7	21.4	53	70	82	92	100
Prunifolium No. 2	9.3	4.9	3.0	1.4	1.0	19.6	47	72	88	95	100
Prunifolium No. 3	9.8	5.7	3.0	1.6	0.8	20.9	47	74	88	96	100
Purnifolium No. 4	9.5	5.3	2.5	1.6	0.9	19.8	46	72	86	94	100

Table III.—Commercial Tree Bark Samples Corresponding to Table II

Drug	Cc. $N/2$ Alkali per 100-Cc. Fraction Distillate						Cumulative % of Total				
	1	2	3	4	5	Total	1	2	3	4	5
Prunifolium No. 1	8.3	4.9	2.7	1.5	1.1	18.5	45	69	86	94	100
Prunifolium No. 2	8.0	3.2	3.1	0.8	0.6	15.7	51	71	91	96	100
Prunifolium No. 3	9.1	4.1	2.6	1.4	0.8	18.0	51	73	88	96	100
Prunifolium No. 4	8.5	4.5	2.4	1.3	0.8	17.5	48	74	89	96	100
Prunifolium rossed bark	10.3	6.6	3.6	2.3	1.8	24.6	42	69	83	93	100

Irregular variations between the *normal* and *iso* forms of the three higher acids are probably due to the fact that both boiling points and solubility in water vary irregularly between the isomers. Mixtures of a higher and a lower acid are detected by the fact that the rate of distillation drops off in the last fractions since the higher acid is practically removed in the first three fractions. Mixtures of formic, acetic and valeric acids, however, can simulate acetic acid as shown by the Viburnum bark distillations given in Tables II and III. Reduction tests indicating formic acid are readily obtained in the Viburnum distillates as well as the odor of the higher acids. The largest amount of acid present seems to be acetic acid.

The *Viburnum prunifolium* barks given in Table II and Table III were representative samples of large commercial lots obtained from four principal dealers, and were authenticated by well-known pharmacognosists.

CONCLUSION

The results given would seem to indicate the following conclusions:

1. Root bark of *Viburnum prunifolium* contains up to some 20% more total volatile acid following hydrolysis than does stem bark.

2. The proportion and kinds of acid present in the mixture are practically the same in the case of root as compared to stem bark. The greater amount present in the former is due to more acid of all kinds rather than more of the higher acids such as valeric. Variation in the distillation rates of root bark differ as widely between individual samples as they do between root and stem bark.

3. One sample of rossed stem bark had more total acidity than did any sample of root bark. A greater proportion of the lower acids seem to be present in this sample, however, than in the unrossed samples or in the root bark, and such treatment may not be wholly desirable.

4. If the amounts and proportions of the mixture of volatile acid present are any measure of activity, there does not seem to be sufficient difference between stem and root bark of *Viburnum prunifolium* to justify that only the root bark be official.

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The Growth Effects of Thiamin Chloride, Ascorbic Acid and Phytohormones on Belladonna and Ricinus*

By Louis C. Zopf†

Data of considerable value are rapidly accumulating from currently widespread experimentation as to the effects of phytohormones; vitamins and related organic stimulants upon seed germination and general growth of plants (1, 5, 7, 8, 13, 15, 17, 18, 20). Most such experiments have dealt with the effects of a single stimulant upon crop plants. Relatively little information is as yet available as to effects of varying combinations of two or more stimulants employed simultaneously or successively. Neither have medicinal plants been employed to any significant extent in such studies.

Most experiments performed by other investigators on the effects of growth stimulants have merely employed seedlings or very young plants. Very few data are available as to the effects of phytohormones and vitamins upon plants permitted to attain maturity.

An experiment was consequently devised to determine the specific effects of certain hormone and vitamin-like stimulants, singly and in combination upon the entire growth cycle of the official species *Atropa Belladonna* and *Ricinus communis*.

EXPERIMENTAL

MATERIALS AND METHODS

The experimental procedure involved (1) the use of vitamin and hormone-like stimulants applied to seeds in various concentrations in a medium of powdered talc (U. S. P.) of 200 mesh in fineness, and (2) the application of aqueous solutions of the stimulants to growing plants in pure quartz sand cultures.

1. Seed Treatment.—Crystalline indole acetic acid and *a*-naphthalene acetic acid were weighed out and added to proper amounts of talc and then made into a uniform mixture by a three-hour treatment in a ball mill. Vitamins B_1 and C were

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prepared as aqueous solutions and measured amounts thereof were added to proper quantity of talc, which was air-dried and then transferred to the ball mill. Vitamin and hormone powders were stored in airtight dark glass screw cap jars. Vitamins were used in concentrations of 10 p. p. m. A similar procedure was followed in preparation of the light-sensitizing eosin dye, "Photosensin," which was used in concentrations of 40 p. p. m.

Lots of thirty seeds each of Belladonna and Ricinus were moistened with a wet cloth, and rolled in the various powder mixtures until coated with a uniform light coating of powder as shown in Table I.

Table I.—Dusting Powders for Seed Treatment for Various Lots

A. 1/1000 of *a*-naphthalene acetic acid.
B. 1/1000 of *a*-naphthalene acetic acid plus 10 p. p. m. of crystalline vitamin B_1.
C. 1/1000 *a*-naphthalene acetic acid plus 10 p. p. m. of crystalline vitamin C.
D. 1/500 of indole acetic acid (heteroauxin).
E. 1/500 of heteroauxin plus 10 p. p. m. crystalline vitamin B_1.
F. 1/500 of heteroauxin plus 10 p. p. m. crystalline vitamin C.
G. 1/1000 *a*-naphthalene acetic acid plus 10 p. p. m. vitamin B_1 plus 10 p. p. m. vitamin C.
H. 1/500 of heteroauxin plus 10 p. p. m. vitamin B_1 plus 10 p. p. m. vitamin C.
I. 1/20,000 of photosensin.
J. 1/20,000 of photosensitizer plus 10 p. p. m. vitamin B_1, plus 10 p. p. m. vitamin C plus 1/1000 *a*-naphthalene acetic acid.
K. Untreated controls.

All seeds were planted in separate wooden sand germinating trays to avoid mingling of chemicals. The Belladonna seeds were planted approximately twice as deep as the thickness of the seed. Castor beans were covered with three-fourths inch of sand. Sand used was sterilized, acid and distilled water washed until free of phosphates. All groups received nutrient and water by daily watering with Knop's (modified) solution (Table II).

Table II.—Composition of Knop's (Modified) Solution

KNO_3	2.0 Gm.
KH_2PO_4	2.0 Gm.
$MgSO_4$	2.0 Gm.
$Ca(NO_3)_2$	8.0 Gm.
H_2O distilled	14000.0 cc.

Seeds were planted April 22nd and kept under ordinary greenhouse conditions. Seedlings were observed every twenty-four hours. Castor beans, because of their rapid rate of germination and growth, were studied for twenty days after the initial sprout was visible. Since the Belladonna seeds were slow in germination and growth, they were observed for twenty-six consecutive days.

Percentage germination (Table III) and rate of germination are shown in (Figs. 1, 2, 3).

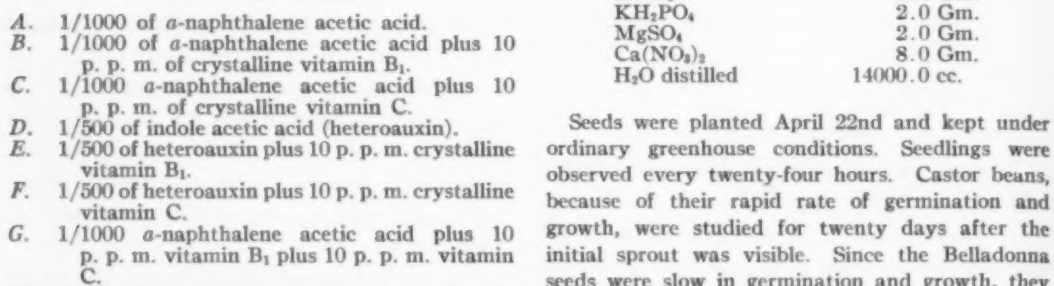

Fig. 1.—*Ricinus communis* Seedlings 20 Days Old (Left) Untreated Control. Slight Inhibitory Effect of 1/500 Indole Acetic Acid Evident in (Center) Indole Acetic Acid Control. Value of Supplementary Vitamin Feeding Exhibited in Indole Acetic Acid 1/500 Plus Vitamin B_1 and Vitamin C 10 p. p. m. (Right).

Fig. 2.—*Ricinus communis* Seedlings 20 Days Old, a Comparative Study of Indole Acetic Acid Control 1/500 (Left). Untreated Control (Center). *a*-Naphthalene Acetic Acid 1/1000 (Right).

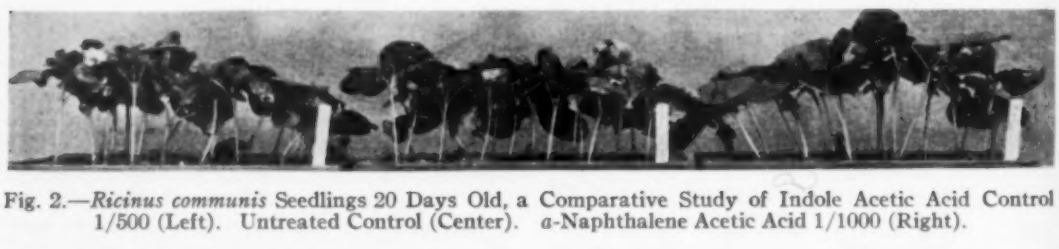

Fig. 3.—*Ricinus communis* Seedlings 20 Days Old. *a*-Naphthalene Acetic Acid 1/1000 Control (Left). *a*-Naphthalene Acetic Acid 1/1000 Plus Vitamin B_1 10 p. p. m. (Center). Improved Growth Effect Upon Addition of the Two Vitamins is Evident (Right).

Table III.—Percentage Germination of *Atropa Belladonna* and *Ricinus Communis* Seeds Receiving Various Dust Treatments

	Atropa Belladonna	*Ricinus Communis*
Control	56.0	73.0
a-Naphthalene acetic acid	66.0	76.0
Indole acetic acid	43.0	86.0
a-Naphthalene acetic acid plus thiamin chloride and ascorbic acid	13.0	93.0
Indole acetic acid plus thiamin chloride and ascorbic acid	13.0	100.0
a-Naphthalene acetic acid plus thiamin chloride	50.0	93.0
Indole acetic acid plus thiamin chloride	56.0	93.0
Indole acetic acid plus ascorbic acid	40.0	90.0
a-Naphthalene acetic acid plus ascorbic acid	53.0	93.0
a-Naphthalene acetic acid plus thiamin chloride plus ascorbic acid plus photosensitizer	46.0	83.0
Photosensitizer	50.0	90.0

2. Growth Study.—For this study Belladonna and Castor Bean seeds were germinated in separate trays containing a 50/50 sandy loam soil. Transplants were made to white sand contained in two-gallon glazed earthenware pots, equipped with side or bottom drain plugs. The initial set of Castor Bean seedlings were planted two to the pot, and an initial set of Belladonna seedlings were planted five to each jar. This group will hereinafter be referred to as Group I.

A second lot (Group II) of both seeds was germinated under the same conditions as Group I. Transplanting was made in the same manner as above with the exception of the Belladonna seedlings which this time were planted two plants to the pot. Group I consisted of two pots of each species to each nutrient series. Group II contained eight pots of each series.

Water and nutrient solutions were applied directly to the sand. The control and basic nutrient solution was Knop's solution (Table II). Thiamin chloride and ascorbic acid, 10 p. p. m. of each, were added to the control solution in the vitamin series *a*-Naphthalene acetic acid when supplied was added as a separate aqueous solution. The initial solution of the acid was made in 10 cc. of alcohol. Nutrient formulas received by the various series are listed in Table IV.

Table IV. Nutrient Formulas[a]

A. Knop's plus *a*-naphthalene acetic acid.
B. Knop's plus *a*-naphthalene acetic acid plus 10 p. p. m. each of vitamins B_1 and C.
C. Knop's plus *a*-naphthalene acetic acid and 10 p. p. m. each of vitamins B_1 and C and 1/25,000 of Photosensin.
D. Knop's plus 10 p. p. m. each of vitamins B_1 and C.
E. Control, Knop's solution only.

[a] NOTE: The vitamin B_1 (Betabion), vitamin C (Cebione) and *a*-naphthalene acetic acid were supplied through the courtesy of Merck & Co., Inc. Indole acetic acid was the product of the Eastman Kodak Co. All chemicals used as nutrients were of C.P. or analytical quality. Distilled water was used exclusively. The photosensitizer was a product of the Photosensin Co.

In addition to the Knop's and other nutrients supplied, each pot received 500 cc. of a solution of minor elements (Table V).

Table V.—Solution of Minor Elements

Solution A	
$FeSO_4$	0.8 Gm.
H_2O	500 cc.
Solution B	
H_3BO_3	0.8 Gm.
$MnSO_4$	0.8 Gm.
$ZnSO_4$	0.8 Gm.
$CuSO_4$ (Crystals)	0.4 Gm.
H_2O *q. s.*	500 cc.

Used 5 cc. of Solution *A* and 10 cc. Solution *B* to each 19 liters of distilled water.

Addition of photosensitizer was made as separate distilled water solutions.

The non-vitamin series of plants all received regular additions of Knop's solution. Equal amounts of Knop's plus the vitamins were administered to the vitamin series. The nutritional dosage was graduated with the age and requirements of the plants. The average weekly amount for a maturing plant was 500 cc. The solution supplying the minor elements was added twice to each pot in amounts of 250 cc. In order to correct and prevent the appearance of chlorosis, each jar including the Castor Bean series received 150 cc. each of 0.1 per cent ammonium sulfate and 10 p. p. m. of iron and ammonium citrate.

In all series receiving *a*-naphthalene acetic acid, the total amount of acid added was 0.0125 Gm. per pot, administered in two doses. The first dose was 0.0025 Gm., and this being without noticeable effect, the next and last dose was increased to 0.01 Gm. per pot.

Immediately following flowering of the Belladonnas the leaves were picked both from the controls and the control vitamin series. They were dried at room temperature for forty-eight hours when they were transferred to an oven and dried at 38° C. Root examination of both Belladonna and Castor Beans were made at this point by flushing sand from the pot.

RESULTS

1. GERMINATION RESPONSES

A. Germination Responses of Ricinus Communis.—Response of *Atropa Belladonna* and *Ricinus communis* seeds treated with the various dusting powders was extremely varied (Table III).

A definite increase in percentage germination of *Ricinus communis* was evident in all seeds receiving the mixture of hormone and vitamin. Hormone treatment alone gave an increase in both *a*-naphthalene acetic acid and indole acetic acid controls over

the untreated controls. A small decrease in *rate* of germination of the Castor Beans was noted upon the addition of vitamins to the *a*-naphthalene acetic acid dust treatment. Percentage germination of the *a*-naphthalene acetic acid plus vitamin series was well above that of the controls.

The only seeds showing 100 per cent germination were those receiving the combined treatment of indole acetic acid, thiamin chloride and ascorbic acid In addition this group exhibited the greatest stem elongation and foliage yield of all groups (Fig. 1). Stem length increases of approximately $^1/_8$ to $^1/_4$ inch over that of the untreated controls are recorded by the indole acetic acid control and the *a*-naphthalene acetic acid control (Fig. 2). In the case of each hormone combined with both vitamins an improved growth response is noted over that of the corresponding hormone control (Figs. 1, 3).

The average growth of *Ricinus communis* seed treated with *a*-naphthalene acetic acid plus thiamin chloride and *a*-naphthalene acetic acid combined with thiamin chloride and ascorbic acid exceeds that of the *a*-naphthalene acetic acid controls by approximately $^3/_4$ and 1 inch, respectively (Fig. 2). Root responses also proved sufficiently definite and varied to justify examination. Results are given synoptically.

B. Root Responses of Germinated Seedlings of Ricinus Communis.—(1) Controls: A definite massed root system with many thick primary roots. Many secondary roots of a coarse hunger type uniformly scattered along the primary roots.

(2) Indole acetic acid: The roots of the series receiving indole acetic acid showed a much finer general root system than either the controls or the *a*-naphthalene acetic acid series. A definite branching occurred primarily at the root tips.

(3) Indole acetic acid plus thiamin chloride: In the case of indole acetic acid plus thiamin chloride the roots showed a vertical stratification due possibly to the vitamin effect.

(4) Indole acetic acid plus ascorbic acid: In indole acetic acid plus vitamin C there was a general massing of primary roots in the hypocotyl region. These primary roots were more slender, less stubby than in the indole acetic acid plus thiamin chloride series, with the whole system showing a horizontal stratification.

(5) Indole acetic acid plus thiamin chloride and ascorbic acid: This series previously mentioned as 100 per cent in germination and excelling in growth, presented in general a smaller root system, in comparison to either of the other series. The primary roots were finer and, as was the case in the other indole acetic acid groups, no tertiary roots were evident.

(6) Photosensitizer: Relatively few stubby primary roots and little secondary branching appeared. The roots were massed in the hypocotylary region.

(7) *a*-Naphthalene acetic acid plus vitamin B_1, plus vitamin C and photosensitizer: A slight thickening and elongation of roots were noticeable in comparison to that of the corresponding indole acetic acid series.

(8) *a*-Naphthalene acetic acid: The roots of the series receiving *a*-naphthalene acetic acid in their treatment present a shorter, less numerous but thicker primary and secondary root system.

(9) *a*-Naphthalene acetic acid plus vitamin B_1: Massing of the roots at the base of the hypocotyl is evident, but not so marked as in the case of the *a*-naphthalene acetic acid plus thiamin chloride and ascorbic acid. A linear stratification of roots is evident.

(10) *a*-Naphthalene acetic acid plus vitamin C: The possible difference in macroscopical examination of these roots is the evidence of the branching along the tap root.

C. Germination Responses of Belladonna.—With the exception of the *a*-naphthalene acetic acid controls, all dust-treated Belladonna seeds gave a lower percentage germination than the untreated controls. The Belladonna seeds treated with indole acetic acid plus vitamin B_1 equaled the untreated controls and surpassed the indole acetic acid controls in percentage germination (Table III).

All dust-treated Belladonna seeds receiving the combination of a hormone and one or both of the vitamins, with the exception of indole acetic acid plus thiamin chloride, show a noticeable retardation in the rate of germination. The controls were among the first to germinate and led in the rate of germination throughout the experiment.

Top growth was so slow and irregular in the treated plants that it is impossible to record the comparative response with any degree of accuracy.

Presented in the order of their best (general) top growth they are as follows: control (untreated), photosensitizer, indole acetic acid plus thiamin chloride, indole acetic acid plus ascorbic acid. The groups presenting the poorest top growth are *a*-naphthalene acetic acid and indole acetic acid plus *a*-naphthalene acetic acid.

D. Root Responses of Germinated Seedlings of Atropa Belladonna.—Extreme irregularities in growth and rate of germination among the various series of Belladonna also made it difficult to study the root system of plants of comparable ages.

In general the root responses of Belladonna could be said to be comparable to that of Castor Bean. All seeds receiving indole acetic acid in their powdered form had a rather extensive but thin secondary root system. The seedlings produced from seeds treated with *a*-naphthalene acetic acid had much shorter and thicker roots. The control (untreated) had slightly less roots than the indole acetic acid series and appeared less fibrous. The roots of the photosensitizer treated seedlings were short but rather numerous. This effect was not noticeable, however, in the group receiving the combined treatment of *a*-naphthalene acetic acid plus vitamin B_1, plus vitamin C and photosensitizer.

2. GROWTH RESPONSES

For uniformity, the plants receiving only Knop's solution will be referred to as the control series. The other series will be designated by abbreviations as follows:

Knop's-*BC*—Knop's plus 10 p. p. m. each of thiamin chloride and ascorbic acid.

Knop's-*BC-P*—Knop's plus 10 p. p. m. each of thiamin chloride and ascorbic acid plus 1/25,000 photosensitizer.

A-N-A-BC—The Knop's *BC* series receiving *a*-naphthalene acetic acid.

A-BC-P—Knop's plus 10 p. p. m. each of thiamin chloride and ascorbic acid plus *a*-naphthalene acetic acid and photosensitizer.

A-N-A—The control series receiving *a*-naphthalene acetic acid.

A. Ricinus Communis.—Five-week-old *Ricinus communis* seedlings (Group I) of the control and Knop's-*BC* series gave no visible response to the addition of 2.5 mg. of *a*-naphthalene acetic acid applied directly to the sand. Again, on the subsequent addition of 10 mg. of *a*-naphthalene acetic acid no immediate responses, such as epinasty of the leaves and bending, were noticeable. Daily observations, however, revealed definite retardation or inhibition of top growth which proved to be a permanent retardation.

Attention is directed to the antidoting effect of the combined vitamins B_1 and C exhibited as increased growth over the *A-N-A* series. Beneficial results from the vitamin additions are prolonged as shown by continued increase in growth through the period of flowering (Figs. 4, 5).

The noticeable response of *Ricinus communis* (Group I) suggested the advisability of the repetition of the treatment in a second group (Group II). In this group a Knop's control, a Knop's-*BC* and a Knop's-*BC-P* series were run, two plants to the pot, eight pots to each series. A general advanced rate of growth was noted in both series receiving supplementary vitamin feedings over that of the controls. The series receiving photosensitizer in addition to the vitamins exhibited a definite increase in stem

Fig. 4.—Illustrating the Antitoxic Value Against *a*-Naphthalene Acetic Acid Toxicity (Left) by Treatment with Vitamins B_1 and C (Second from Left); (Second from Right) Knop's Control; (Right) Vitamin Control.

Fig. 5.—*Ricinus communis* (Group I). Toxicity of *a*-Naphthalene Acetic Acid (Left) on Top Growth and Antidoting Effect of Vitamins B_1 and C (Center). Knop's Solution Control (Right). Above Are the Same Plants as Shown in Fig. 4.

elongation and growth rate above those receiving Knop's plus the vitamins. Size and number of leaves were comparable in all series. Simultaneous blossoming was also noted. Difference in top growth was pronounced (Fig. 6).

Flowering Ricinus Communis—Root Growth.—Root studies of the two groups immediately after flowering are shown synoptically below.

Knop's Control: Abundant number of thick, fairly long primary roots. These branch from the hypocotyl region. Secondary roots are fairly coarse but very abundant branching is also evident from tap root.

Knop's-*BC:* This series shows a similar number of primary roots as compared with Knop's control, branching from the hypocotyl region. A finer system of secondary roots is in evidence and they appear functional.

A-N-A: The branching primary roots of this series are spotted with a white warty-like excrescence of the same appearance as that in the *A-N-A-BC* group. Primary roots are smaller than in the *A-N-A-BC* group. Secondary roots coarse. Noticeable because of its absence is the usual outstanding tap root.

All plants seem to show a large top root ratio.

B. Atropa Belladonna.—Since the controls and Knop's-*BC* series of Belladonna of Groups I, II and III responded fairly uniformly in each series these will be mentioned first.

The three-inch seedlings of *Atropa Belladonna* receiving Knop's-*BC* gave the most vigorous early growth responses.

Also evident in this series was the better leaf color and less anthocyanin pigmentation of the stems. The early rapid growth of the Knop's-

Fig. 6.—*Ricinus communis* (Group II). Top Growth Response of the Vitamins B_1 Plus C (Center) Over Knop's Control (Left). Increased Stem Elongation of Vitamin Treated Plants Plus Photosensitizer (Right).

A-N-A-BC: This series presents the heaviest root system. Secondary branching occurs close to the soil line and appears fibrous, with an abundance of such secondary roots are present toward the base of the pot. A white excrescence is evident along the primary roots of this series.

Knop's-*BC-P:* This series has the smallest roots of either group, fairly uniform primary root branching is evident from the hypocotyl region. The primary roots are approximately two-thirds the size of those of the control. There is a much finer anastomozed branching system of secondary roots. The latter are in abundance but much less fibrous than those of other series. No excrescence is evident in this system.

BC series seemed to cease after the plants had acquired a few leaves and a height of approximately eight inches. From this point these plants were overtaken by the controls in growth.

Typical flowering plants from the Knop's-*BC* series show a slightly improved top growth and deeper green color over plants of the same age in the control series (Figs. 7, 8).

Further growth responses were noticeable but difficult to evaluate definitely. Earlier and more universal basal branching was produced in the Knop's-*BC* group (Figs. 7, 8). Control group did exhibit some of this type branching but in relatively few plants. Blossoming occurred simultaneously in both series at twelve weeks of age. No difference

Fig. 7.—Flowering Belladonnas (Group II) of the Knop's Control Series, Age Twelve Weeks.

Fig. 8.—Flowering Belladonnas (Group II) Receiving Vitamins B_1 and C 10 p. p. m. in Addition to the Knop's Solution. Note Basal Branching and Deeper Green Color. Age Twelve Weeks.

in duration of period of flowering or number of blossoms was noticed.

In general, the Knop's-*BC* series gave the least evidence of chlorosis, though this did occur in both series demanding supplementary feeding of the ferric ion as 10 p. p. m. of ferric and ammonium citrate and additional nitrogen through a 0.1 per cent solution of ammonium sulfate.

Immediately after blossoming both series of plants were stripped of their leaves which were oven-dried for future alkaloidal assay.

The series of Belladonna seedlings receiving *a*-naphthalene acetic acid in addition to their regular nutrients represented a most interesting study. Each of the three series in this group received the same concentration and dosage of *a*-naphthalene acetic acid. As was noted in the Castor Bean seedlings, the initial addition of 2.5 mg. *a*-naphthalene acetic acid per pot gave no visible response. Within a period of twelve hours following the next and last addition of *a*-naphthalene acetic acid, 10 mg. per pot, a definite epinastic and bending response was noted. The toxic effect shown by defoliation was most noticeable in the control series.

First to revive from this toxic dose were the two series which received vitamins in their nutrient. The most rapid and energetic response was shown by that group receiving *A-N-A-BC* and *A-BC-P*. Greater top and root growth is very evident in the plants receiving vitamins. New shoots arose from old stems in the *A-N-A-BC* group (Fig. 9). The figure also indicates the degree of the *a*-naphthalene acetic acid toxicity.

Mention has been made of the fact that the phyto-

Fig. 9.—The Antidoting Effect of Vitamins B_1 and C (Center) Against *a*-Naphthalene Acetic Acid Toxicity on Roots (Left). Improved Response (Right) Through Addition of Photosensitizer Plus the Vitamins. Plants of Like Age.

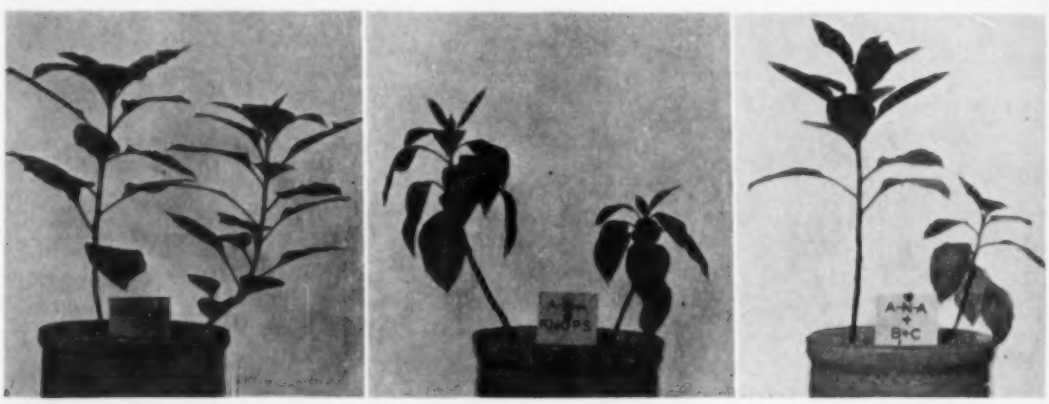

Fig. 10.—The Epinastic Response of *a*-Naphthalene Acetic Acid (Center) on Belladonna of the Knop's Control Series. Retarded or Delayed Epinastic Response in Belladonna Plants (Right) of the Same Age Receiving the Same Dosage of *a*-Naphthalene Acetic Acid, Subsequent to Knop's-BC. Untreated Control (Left).

hormones are more toxic to young than mature plants. With this thought in mind, Belladonna plants just about to flower having been treated up to this time with Knop's and Knop's-*BC*, respectively, were given a dosage of 10 mg. of *a*-naphthalene acetic acid per pot. Figure 10 shows the epinastic response which was induced in the plants of both series but which is much more pronounced in the control group.

The Knop's-*BC* plants receiving 10 mg. of *a*-naphthalene acetic acid did not show as marked epinastic responses to *a*-naphthalene acetic acid and exhibited recovery sooner than the controls with *a*-naphthalene acetic acid but without the vitamins. Lower leaves on both plants dried completely but at no time during the period of two weeks after the toxic dose was administered did either group lose all leaves. Those receiving the Knop's-*BC* were always more vigorous than the control.

DISCUSSION

The foregoing results coincide with those of other workers in showing (1) a toxic effect of *a*-naphthalene acetic acid (7, 18, 25); (2) a beneficial effect of vitamins on plant growth (2, 3, 4, 5, 6, 11, 12, 13) and (3) variable effects of those stimulants upon seed germination (8, 11, 12, 13, 19). Treatment is beneficial in many, though not in all cases (8, 13, 19), the effect varying with

concentration and different combinations of stimulants.

The variability in germination of *Ricinus communis* and *Atropa Belladonna* in response to a given stimulant suggests wide differences between species, as also noted by other investigators (5, 13, 20), especially as to optimal concentrations of the added chemicals.

It is obvious that seeds slow to germinate, such as *Atropa Belladonna* and others, require a powder treatment of lower concentration, even though growth substances are released slowly, as shown by Grace (8). Grace, experimenting with two dusting techniques, which she generally found to be safer than treatment with solutions, used talc or a standard mercurial dust disinfectant for treatment of seeds of wheat and barley. In Grace's experiments concentrations of 5 p. p. m. of growth substances were found to be effective in many instances.

Literature dealing with the growth effects or response of plants to the water-soluble vitamins is limited. Von Hausen experimented with the absorption and germination effects of ascorbic acid by pea seeds. She found solutions of ascorbic acid in higher concentration than 0.4 per cent could not be used without a serious loss of the germinating power of the seeds. Havas (13) using vitamin C in a solution for seed treatment also noted a delay in germination in solutions above a certain concentration. Optimum growth conditions were obtained by use of vitamin B_1 by Robbins and Bartley (20) working with excised tomato root tips in artificial nutrient cultures. The work of Bonner (4) and Bonner and Greene (5) indicate the value of vitamin B_1 in the form of improved root growth. They found vitamin B_1 to be not so much for the initiation of the root primordia as for their development. The present investigation correlates well with the foregoing in showing the value of simultaneous administration of the two vitamins B_1 and C to the soil of plants in inducing increased root growth and improved top growth (Figs. 5, 6, 7, 8, 9, 10). These vitamins also display an antitoxic effect upon the toxicity resulting from *a*-naphthalene acetic acid (Figs. 5, 9, 10).

It has been demonstrated by Loehwing and Baugess (17) that the application of héteroauxin nutrient solutions directly to the soil result in stimulation of early growth. Other hormone substances, likewise, have been applied to the soil (14) and found to induce all of the responses obtained by applying the material to the aerial parts of plants in the form of a lanolin paste. Absorption of growth substances in this experiment is evidenced by (1) the inhibitory growth response by the *Ricinus communis*, (2) through the delayed or inhibited germination of the *Atropa Belladonna*, (3) the marked epinastic response of the Belladonna plants and (4) the definite antitoxic responses exhibited by supplementary feeding of the two water-soluble vitamins.

The fact that the antidoting or antitoxic value of the vitamins is not ascribable to a chemical combination of the hormone with the vitamins, thus perhaps rendering either or both inert or unavailable to the plants, is demonstrated by the increased growth of the plants in the *A-N-A-BC* series over that of the controls (Fig. 5). The epinastic response which was manifest upon the administration of 10 mg. of *a*-naphthalene acetic acid to Belladonna plants just prior to anthesis shows the comparatively low threshold which these plants have for this particular hormone. The small amount of epinasty in the vitamin-treated Belladonnas later became more severe than pictured (Fig. 10). The vitamins served to retard the epinasty of the leaves both in onset and duration. Tobacco, also a member of the *Solanaceæ* (Nightshade Family), when treated with *a*-naphthalene acetic acid (14) induced epinasty of the leaves but only with larger dosage. This serves as an illustration of the varying hormone threshold of plants in the same family. Differences in species of plant, concentration of hormones and method of hormone application are not only important but even crucial in investigations of this sort (12).

It was observed that the retarded growth rate of the *Ricinus communis* in the *a*-naphthalene acetic acid control was permanent. No marked increase in growth rate was noticed following the original re-

tardation and, though the plants continued to grow, at no time did they equal the regular controls in stature (Figs. 4, 5). If the vitamins B_1 and C exhibit both such stimulatory effects and antidoting properties toward other pharmaceutical and economic plants, their action is well worthy of further study. The value of earlier germination, increased growth (top and root) and early maturation of official and agricultural plants is obvious.

CONCLUSIONS

1. Hormone and hormone plus vitamins (B_1 and C) as dust treatment of seeds influence both germination and growth responses of *Atropa Belladonna* and *Ricinus communis*. The effect of a given stimulant, whether beneficial or injurious, varies with its concentration, the species and age of the plants.

2. *a*-Naphthalene acetic acid applied as a solution in toxic dosage induces varying response in different plants. The toxic threshold varies with the type of plant.

3. The simultaneous administration of vitamin B_1 and vitamin C exhibits a combined effect on the general growth of the plants studied, which is more than a mere additive stimulus.

4. The simultaneous administration of vitamin B_1 and vitamin C exhibits an antitoxic or antidoting property toward plants which have previously been subjected to toxic doses of hormones such as *a*-naphthalene acetic acid. This antitoxic reaction (occurring as improved top and root growth) is not ascribable to a neutralizing effect or chemical combination between the *a*-naphthalene acetic acid and the vitamins, as evidenced by the better top growth of the *A-N-A-BC* plants over the controls.

5. The antitoxic quality of concurrent administration of the water-soluble vitamins B_1 and C is also of value in delaying and reducing the degree of epinastic responses of Belladonnas about to flower.

6. Photosensitizer added directly, in aqueous solution, to the soil of plants containing both vitamins B_1 and C, produces increased top growth.

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A Study of the Stability of Liquid Preparations Containing Pepsin*

By C. J. Klemme† and C. L. Boswell‡

This work is an endeavor to study the stability of liquid preparations containing pepsin, and to investigate the possibility of stabilizing the activity of peptic systems. Heretofore, as mentioned by Worrell (1), stability studies of pepsin preparations have been inconclusive, due primarily to the lack of a satisfactory method of assay. Worrell (1) had this objective in mind, namely, to develop a procedure which could be employed successfully in the accurate assay of pepsin preparations for activity. In the experiments conducted in this research his method of assay for liquid preparations containing pepsin has been used entirely.

During the course of making an enzyme preparation, it is often noted that there is a rather acute loss in activity of the enzyme. Similar losses are noted when the enzyme solution is permitted to stand at room temperature for a period of time. Northrop (2, 3) indicates that a major source of inactivation in the case of pepsin and trypsin is due to denaturation of the enzyme protein.

In attacking the problem of stabilizing the activity of liquid preparations containing pepsin, it was deemed fundamental that a study first be made of the influence of individual factors or variables on the maintenance of peptic activity over an extended period of time. The factors which have received special consideration include temperature, p_H, antioxidants (maleic acid, hydroquinone, resorcinol), preservatives (alcohol, "Merthiolate"), protective agents (acacia) and low concentrations of amino acids (tyrosine).

EXPERIMENTAL

A number of preparations were made, each having one or more of the above variables which differentiated it from the standard. A standard preparation was designated as one having approximately five per cent of pepsin in distilled water, and which was stored at room temperature under nitrogen. Any substance added in order to study its effect on the stability of pepsin solutions was considered to be a variable. All of the samples were placed in ordinary clear glass bottles and stored in the dark.

Table I.—A Summary of Assay Results of Pepsin Solutions on Stability Tests. ("*n*" in the Number Designating the Sample Indicates Storage under Nitrogen; "*a*" Indicates Storage under Air)

Sample	Variable	Concentration of Pepsin (%) 1st Week	6th Week	14th Week	% Loss in Activity
3*n*	Storage at 5° C.	5.4	5.0	4.9	9.3
4*n*	1% maleic acid	5.2	3.4	2.5	51.9
5*n*	3% maleic acid	4.8	3.0	1.9	60.4
6*n*	5% maleic acid	4.8	2.5	1.3	72.9
7*n*	2% resorcinol	5.5	4.5	4.6	16.4
8*n*	2% hydroquinone	5.3	4.2	2.8	47.2
10*n*	10% U. S. P. Alcohol	5.7	4.8	4.9	14.0
16*a*	0.05% "Merthiolate," storage under air	4.6	4.4	4.2	8.7
19*n*	0.05% "Merthiolate"	4.5	4.0	4.1	8.9
21*n*	0.1% maleic acid, 10% U. S. P. Alcohol	5.3	5.2	4.7	11.3
22*n*	10% U. S. P. Alcohol	5.2	5.0	4.7	9.6
24*n*	0.05% "Merthiolate"	5.4	4.9	4.5	16.7
25*n*	0.05% "Merthiolate," 0.1% maleic acid	5.7	4.8	4.5	21.1
26*n*	0.05% "Merthiolate," 1% acacia	5.3	4.9	4.6	13.2
27*n*	0.05% "Merthiolate," satd. with tyrosine	5.3	4.9	4.6	13.2

* An abstract of a thesis submitted to the faculty of Purdue University in partial fulfilment of the requirements for the degree of Master of Science.

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Assays were made weekly over a period of ten weeks on these preparations. An assay of the samples was also made a month following the ten-week period. A summary of the results of the assays is included in Table I.

Following these assays to determine the effect of storage on the activity of pepsin exposed to various factors, an experiment on the effect of shaking pepsin solutions was carried out.

A stock solution of pepsin approximately five per cent in strength was prepared. Portions of the stock solution were placed in several bottles to be shaken for various periods of time. For the purpose of finding out whether or not the effect of shaking was associated with air oxidation, identical samples were also stored under nitrogen. The stock solution was assayed at the beginning of the experiment, and twenty-four hours later each of the samples was assayed to determine the effect of shaking on the proteolytic activity.

Table II.—The Effect of Shaking on Pepsin Solutions

Pepsin lot 220799, Casein lot 33618

Sample	Period of Shaking	Gm. Pepsin per Cc. before Shaking	Gm. Pepsin per Cc. after Shaking	% Loss in Activity
Aa	1 hour	0.0488	0.0458	6.1
An	1 hour	0.0488	0.0462	5.3
Ba	2 hours	0.0488	0.0458	6.1
Bn	2 hours	0.0488	0.0462	5.3
Ca	6 hours	0.0488	0.0462	5.3
Cn	6 hours	0.0488	0.0462	5.3
Da	12 hours	0.0488	0.0441	9.8
Dn	12 hours	0.0488	0.0441	9.8
Ea	24 hours	0.0488	0.0387	20.7
En	24 hours	0.0488	0.0387	20.7
Fa	Not shaken	0.0488	0.0462	5.3

CONCLUSIONS

In general, it appears that the rate of loss in activity is greatest during the first six weeks of storage. The advantage of refrigeration is not sufficient to warrant its consideration commercially.

The addition of maleic acid presumably as an antioxidant leads to a marked loss in activity, the degree of inactivation paralleling increasing concentrations of the acid. Solutions containing sodium maleate were not included in the table of results because of putrefaction early in the assay period. However, the sample containing three per cent of sodium maleate was sufficiently alkaline to completely inactivate the pepsin by the end of the first assay.

Thus, it is obvious that ionization markedly influences the activity of pepsin. The presence of hydroxyl ions induces an immediate inhibition of pepsin. Hydrogen ions in excess also promote a gradual loss in activity. Apparently the optimum p_H for the storage of pepsin solutions is in the range of 4.5 to 5.0.

The antioxidant action of resorcinol and hydroquinone is of little value in preserving enzymatic activity. Both of the solutions containing these agents darken upon standing.

Alcohol and "Merthiolate" in the concentrations employed exhibit no discernible inhibition of peptic activity. The use of acacia as a sorbing or protective agent appears to be of no value. Saturating a pepsin solution with tyrosine apparently has no activating effect.

Vigorous shaking for a period of six hours or less produces no increased loss in activity of pepsin in a purely aqueous solution. However, it is noted that shaking for a period of twelve hours or longer results in a marked inactivation of the enzyme.

Storage under an atmosphere of nitrogen does not retard loss in peptic activity. From the experiment on the effect of shaking, samples stored under nitrogen (denoted by the letter "*n*") act the same as those stored under air. As a direct consequence, it appears that air oxidation has little to do with the inactivation of pepsin.

It is evident from these results that protein denaturation is primarily responsible for loss in peptic activity. Factors which promote protein denaturation also produce inactivation of pepsin. Apparently, the presence of water is conducive to protein denaturation or loss in activity.

A logical extension of this work is being made in an attempt to develop a pepsin preparation which will retain its initial activity over a long period of time.

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Cultivation of Fennel in Washington

*By Troy E. Becker, Edwin J. Isler and Forest J. Goodrich**

Because of the economic and pharmaceutical importance of the fruit and volatile oil of *Fœniculum vulgare*, Miller, and since they are largely imported into the United States, cultivation of the plant has been conducted for several years in the College of Pharmacy drug garden. The character and quality of the products have been determined and comparisons made with the imported fruit and official oil. It is interesting to note that fennel has been cultivated for the past ten centuries in the old world. Plants have been grown in this country but not in sufficient quantities to meet the demand for the fruit and volatile oil.

EXPERIMENTAL

Description of Plant.—Large perennial herb attaining a height of five and one half to six feet. The stem is furrowed, glaucous, green and branched. Leaves are alternate, twice pinnate with narrow pinnea. The root stock is thick, the flowers a pale yellow and arranged in a compound umbel. The fruit is a large cremocarp. All parts of the plant are aromatic.

Planting and Collection of Fruit.—The soil was a good sandy loam with considerable limestone. The plantings were made in March in rows eighteen inches apart. The plants were later thinned to about twelve inches apart in the rows. No fruit was obtained from the first year's planting. The latter part of September of the third year, the fruiting branches were cut off and the ripe fruit threshed, screened and allowed to dry. The yield of air-dried fruit was slightly under two tons per acre, 3960 pounds.

Extraction Ash and Oil.—Several pounds of the fruit were ground to approximately a number forty powder and reserved for experimental use.

Successive extractions of forty gram samples gave the following percentage extractions:

Solvent	Extractive
Petroleum ether	19.62
Ethyl ether	2.31
Chloroform	2.29
Ethyl acetate	3.01
Ethyl alcohol	4.70

Two-gram samples of the powdered fruit were ignited to constant weight and the following determinations made:

Total ash	7.14
Water insoluble ash	4.32
Acid insoluble ash	0.60

Several pounds of the ground fruit were macerated and steam distilled until all of the oil was carried over. The following results were obtained:

Yield of Volatile Oil	4.1%
Properties of Oil	
Sp. gr. at 25° C.	0.9641
Soluble, 10 parts 80% alcohol	
Not entirely sol., 1 part 90% alcohol	
Optical rotation	−14.1°
Refractive index 20°	1.534
Odor, strongly fennel-like, somewhat pungent	
Color, pale yellow	
Congealing point	2.9° C.
Heavy metals test	negative
Appearance, bright	
To litmus, slightly acid	
U. S. P. Requirements for Oil of Fennel	
Sp. gr.	0.953/0.973
Solubility	8/80%
Solubility	1/90%
Optical rotation	−12°/24°
Refractive Index 20°	1.5280/1.5380
Odor, fennel-like	
Color, pale yellow	
Congealing point	3° C. or above
Heavy metals test	negative (approx.)

The solubility in alcohol and the congealing point is slightly below U. S. P. requirement, otherwise, the oil meets the requirements of the U. S. P. XI by all constants and tests.

Histological sections were made of the fruit and comparisons made with species grown in Europe. The Washington-grown fruits are somewhat lighter in weight and not as full and plump as some European samples examined. The Vittæ in many cases seem to be somewhat more ellipsoidal.

Considering the large yield of American-grown fennel and the fair quality of oil, it would seem worth while to develop *Fœniculum vulgare* cultivation in this country, at least in this section of the country where growing properties have been established.

* University of Washington College of Pharmacy. Presented before the Scientific Section, A. PH. A., Richmond meeting, 1940.

A Permanently Stable Elixir of Ferrous Sulfate*

By Donald A. Clarke†

It is the purpose of this report, not to disclose any startling discoveries, for such have not been made, but merely to recount a simple study which has been conducted on the preparation of an elixir of ferrous sulfate

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so that other individuals who might be faced with a similar problem may find a resolution in the products of our efforts.

Through the medium of the Formulary Committee of The New York Hospital, we were requested to supply various clinical services of the hospital with a liquid preparation of ferrous sulfate which would lend itself readily to the administration of ferrous sulfate to children, or for which dosage schedules, when necessary, could be made more flexible than tablets of the salt would allow.

Due to the fact that the required dose of any particular iron preparation to be used for the treatment of iron deficiency is proportionately smaller for infants and children than the dose for adults, a liquid preparation of a good iron salt is needed for treating the range of patients from newborns to adults to allow flexibility in administration. Also, it is often impossible to administer a tablet containing a definite quantity of such a salt to infants and children either because of incapability of swallowing such a tablet or, due to preconditioning, outright refusal to take a tablet. The latter circumstance is also found true in the case of some adults. These preconditioned cases, however, will often take a good appearing, well flavored, liquid preparation. Such a preparation of a ferrous salt must be permanent indefinitely and must retain its original appearance indefinitely under all practical conditions of use. No dissertation is necessary to point out the obviousness of these two fundamental requirements.

The development of a formula and a technique for such a preparation of ferrous sulfate was almost entirely one of "feeling in the dark." We were convinced that any available proprietary preparation was not satisfactory from the standpoint of our requirements because rapid decomposition and precipitation of what appears to be ferric hydroxide occurs when exposed to air (repeated openings of the bottle) or direct sunlight. A formula reported by Mackay and Jacob (5), which they stated would give a preparation which would remain stable for about sixty days, gave us, at the most, six to seven days stability. Various antioxidants were used by these authors to retain the ferrous iron in that state. Other than this one report, we could find nothing in the literature of apparent value. We, therefore, proceeded on a trial and error basis.

We found it helpful in approaching the problem to first exhaust all simple and usually well-known possibilities. In this instance our answer apparently lay in the use of one of the simplest agents available. Knowing that a small amount of sugar will usually protect the ferrous ion from oxidation for a short period of time in certain preparations of ferrous salts, we theorized that the amount of oxidation of ferrous sulfate in solution over a period of time should be inversely porportional to the concentration of sugar present in the same solution and, likewise, the length of the period of protection should be directly porportional to the amount of sugar present. On the basis of this theory, we felt that there must be a point where a concentration of sugar would be adequate to protect completely and indefinitely a given quantity of ferrous sulfate from oxidation into a ferric state.

EXPERIMENTAL

It was requested that this liquid preparation of ferrous sulfate have a strength of 0.1 Gm. of the salt per 4 cc. Using 4.6 Gm. of U. S. P. XI Ferrous Sulfate per 100 cc. (based on the U. S. P. XI minimum content of 54.36% $FeSO_4$, this is equivalent to 0.1 Gm. of Exsiccated Ferrous Sulfate per 4 cc.) we found that after 24 hours of normal laboratory conditions (no direct sunlight) the amount of ferric ion present was roughly proportionally smaller in samples containing increasingly larger percentages of sugar; *i. e.*, 10%, 20%, 30%, 40%, 50%, 60%, 70% and 80%. This rough comparison was established using an 80% sugar concentration as standard and precipitating the ferric ion present with potassium ferrocyanide test solution. Later, a series was studied using potassium thiocyanate 3*N* solution as a reagent for producing a characteristic color in the presence of the ferric ion. All preparations were compared against a standard solution containing a known quantity of ferric iron by an electrocolorimeter. The results of this study, when plotted, substantiated our theory. The curve plotted out slightly above the concentration of a saturated solution of sugar in water, *i. e.*, 85%; however, we are inclined to believe that the ferric iron present in an 85% sugar preparation is almost entirely ferric iron occurring in the salt before in-

corporation. Nevertheless, we concluded that our optimum preparation should be one using simple syrup as the solvent. Following the curve in such a preparation, it was felt we might rightfully expect either the presence or formation of some ferric iron to be indicated. How long before this formation of ferric iron would become apparent to the eye could only be determined by trial. We found that by adding a sufficient quantity of simple syrup (definitely saturated) to the required amount of ferrous sulfate to make a given quantity of preparation and then triturating in a mortar to grind the crystals, the salt would dissolve readily. For large quantities, the weighed salt was ground into tiny particles under a small quantity of simple syrup, the mixture transferred to a graduated glass-lined vat and sufficient simple syrup used in rinsing the mortar to make the desired volume. Solution was effected by agitation with a mechanical stirrer.

Spirit of Peppermint was used to flavor this preparation for three main reasons. By consensus of opinion, the flavor of peppermint is accepted to be the most popular among all peoples, and especially among children. The menthol contained in peppermint has a slight anesthetizing effect on the taste buds of the tongue. In the concentration used it was found to completely cover the disagreeable ferrous taste inherent in a similar preparation without flavoring. And, finally, it can be stated that the designation "elixir" might be open to question because this preparation does not necessarily conform to such terminology. Because the title, elixir, has been popularized commercially for this type of liquid ferrous sulfate preparation, we were faced with the necessity of making a preparation which could be dispensed when an "elixir" was prescribed or of changing the conventional terminology in the minds of some five hundred staff physicians who might prescribe this medicament at The New York Hospital. Because of the desire to assure the use of our preparation, we, therefore, added a slight amount of alcohol in the form of Spirit of Peppermint to make this liquid one which could at least be described theoretically as being hydroalcoholic and fit the designation, "elixir."

The observable stability of this preparation was found to be a period of three to four weeks, at the end of which time slight precipitation of ferric hydroxide was apparent, especially if the control container had been opened several times or was at all exposed to direct sunlight. As noted previously, we believe this is due to the presence of the ferric ion in the ferrous salt when purchased. It was therefore decided to increase slightly the acidity of the preparation by use of a small amount of citric acid. Upon the addition of 0.2% citric acid, our objective was achieved. The original control bottles, taken from the first lot treated, are now nearly ten months old, have been subjected to every conceivable condition of storage, including prolonged exposure to air and direct sunlight and exposure to high and low temperatures. The samples thus treated retained the same appearance in every respect as control samples stored under optimum conditions, the latter being of the same appearance at the time of this writing as originally. This preparation, the formula for which follows, can therefore be considered permanent for all practical purposes.

Elixir of Ferrous Sulfate

Ferrous Sulfate, U. S. P. XI	4.6 Gm.
Citric Acid	0.2 Gm.
Spirit of Peppermint	0.2 cc.
Simple Syrup, U. S. P. XI. *q. s.*	100.0 cc.

CLINICAL EVALUATION

Clinically, the preparation has proved effective. The Department of Pediatrics of The New York Hospital has studied the effectiveness on iron deficient children who had received no therapy previous to admission. Adequate response was noted in from three to four weeks as determined by the increase in hemoglobin content of the blood.

COMMENTS

It is pertinent to note that the more fresh the supply of ferrous sulfate, the less the amount of the ferric ion present. No crystal of ferrous sulfate is free from ferric ions unless it has been prepared and stored under an atmosphere devoid of all oxygen or oxidizing agents.

A peculiar phenomenon occurs in the elixir made from relatively old samples of the salt. When made from a fresh salt, the elixir is almost colorless, except for a very slight bluish, fluorescent-like color which can be seen through transverse light. Elixirs made from older samples of the salt develop a light green color about an hour after manufacture, which becomes increasingly noticeable the older the sample of salt used. This color is apparently due to the presence of citric acid. When an elixir, which has developed this light green color, is subjected to actinic light, the color almost immediately disappears, but when removed from exposure, it regains its color within a short time. It is important to note at this point that any green color which develops in preparations made according to the formula given above does not increase in intensity after prolonged exposure to light, nor does the original amount of ferric ion increase so that a change can be detected colorimetrically as described earlier.

After this study had been completed and the elixir was undergoing clinical evaluation, various and sundry iron preparations of this type made by private formulas were brought to our attention. We examined a number of these preparations from a comparative standpoint. One such preparation makes use of the principle for stabilizing ferrous salts which we found satisfactory. The formula for this preparation calls for Ferrous Sulfate 3.5 Gm., Alcohol 12 cc., Citric Acid *q. s.* for solubility and Distilled Water *q. s.* to make 100 cc.

In preparing samples of this preparation for examination, we used the same quantity of citric acid employed in our preparation, *i. e.*, 0.2%. The finished preparation remained practically colorless for about one hour after manufacture, at which time a pronounced green color began to develop. The appearance of the green color, which became progressively intense, was taken as evidence of an increasing amount of ferric iron in the preparation. The preparation, examined colorimetrically at intervals revealed that the amount of ferric iron increased until precipitation occurred. When exposed to ordinary daylight with about two hours of direct sunlight per day on intermittent days when the sun shone, the preparation became colorless after a short time. With the passing of daylight, the green color recurred with greater intensity than was the case before the period of exposure. This continued until after an average of about eighteen days when the preparation turned purple, appeared colloidal upon examination, and gave the tyndal cone effect with transverse light. Twenty-four hours later a precipitate of what appeared to be ferric hydroxide began to form and continued until a large portion of the iron salt was precipitated.

Other preparations examined behaved in similar manner. All showed decomposition after varying periods of exposure to actinic light, air, cold or heat, a combination of the four agents or any two or three of them.

CONCLUSIONS

Using a simple approach, we have developed a formula for an elixir of ferrous sulfate which appears to us to be permanently stable. This preparation appears, likewise, to have all the desirable features of such an iron preparation with but few of the undesirable features. It incorporates a form of iron which, clinically, meets all the requirements necessary in treating the typical iron deficiency anemias but having the undesirable feature of possible gastro intestinal upset—a characteristic of all iron preparations commercially available today. Chemically, it meets all the requirements needed to insure its stability. We have found a minimum amount of ferric ion present after manufacture and the quantity does not increase perceptibly after long periods of storage. It is appealing to the esthetic senses. Thus, pharmaceutically, it is, in our estimation, near perfection.

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Hydrogenated Castor Oil as an Ointment Base

V. Jellified Ointments*

By George W. Fiero†

For certain types of ointments, an occlusive ointment base is desired. The most commonly employed occlusive ointment base is petrolatum. Unfortunately, this base has been used to a large extent without regard to its occlusive properties largely because of the fact that it is stable and has the proper consistency. For certain purposes, a liquefying gelatinized ointment base would be desirable.

In a previous paper (1), the writer discussed the properties of salts of hydroxystearic acid obtained from completely hydrogenated castor oil. This fatty acid has been found to have the peculiar property of producing solid gels when dissolved in oils, either liquid petrolatum or fixed oils, and hydrocarbons such as petroleum distillates, oil of turpentine, etc. By varying the amount of the acid, the consistency of the gel may be governed, and gelatinized ointments of various types may be obtained.

Cosmetic products prepared with liquid petrolatum and hydroxystearic acid possess desirable qualities of softness whether emulsified or non-emulsified, liquid or solid. Thus cold creams containing hydroxystearic acid have a soft jelly-like consistency which is highly desirable.

Liquefying creams can be obtained by

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substituting the hydroxystearic acid for part or all of the wax or other solidifier. Brilliantines can be prepared from these jellied oils.

EXPERIMENTAL

The hydroxystearic acid[1] was obtained by saponification of practically completely hydrogenated castor oil. It was found to have the following characteristics:

Acid value	185.9 mg. KOH
Molecular equivalent	299.5
Molecular weight of hydroxystearic acid	300.28

Because of its high melting point it was found more satisfactory to prepare a stock solution consisting of one part of the acid with four parts of heavy liquid petrolatum. This could be readily melted and diluted with liquid petrolatum when desired.

The heavy liquid petrolatum (white mineral oil) had the following specifications:

Specific gravity	0.875–0.885
Color	30 upward
Viscosity at 100° F. (Saybolt)	200–210
Flash point	360° F.

Consistency.—Since the consistency of the jellified liquid petrolatum varies with the content of hydroxystearic acid, various concentrations were prepared and the consistency was determined. The relative consistency was determined by the penetration method described in a previous paper (2). This consists of measuring the penetration in millimeters of a 1.6 cm. disc into the ointment in sixty seconds with varying added weights. White mineral oils[2] of different viscosities were employed; the characteristics are shown in Table I.

Table I.—Mineral Oils Employed

No.	Specific Gravity at 15.5° C.	Sayboldt Vis. at 43.3° C.	Kinematic Vis. at 37.8° C.
1	0.885–0.895	335–345	0.735–0.757
2	0.880–0.890	320–330	0.702–0.724
3	0.875–0.885	200–210	0.437–0.460
4	0.875–0.885	175–185	0.381–0.404
5	0.875–0.885	125–135	0.265–0.290
6	0.850–0.860	80–90	0.156–0.182
7	0.835–0.845	65–75	0.117–0.144
8	0.830–0.840	50–60	0.074–0.103
U. S. P. Heavy	0.860–0.905 (25°)	...	>0.381
U. S. P. Light	0.828–0.880 (25°)	...	<0.370

Gels were prepared by dissolving the hydroxystearic acid in the hot mineral oil and allowing to cool. After standing several days at room temperature (about 23° C.), the consistency was determined. There was no trace of bleeding in the one per cent samples of any of the U. S. P. heavy or light oils (Nos. 1–6). The very light oils did not bleed with two per cent of the acid, but the one per cent gel bled a few drops in the case of No. 7 and about 1 cc. of a 50-cc. sample in the case of No. 8. The relative consistency is shown in Table II.

Official Ointments.—Jellified liquid petrolatum was found to be compatible with materials which are compatible with petrolatum and the resulting ointments were quite satisfactory. They possessed the advantage over petrolatum that they liquefied when applied to the skin, but remained solid at ordinary temperatures. The amount of hydroxystearic acid varied with the consistency desired and with the medicament. For example, in ointments containing considerable volatile oil, such as compound menthol ointment, more hydroxystearic acid was necessary.

The method of preparing the ointments consisted of dissolving or incorporating the medicament with the liquid petrolatum and adding the molten 20% solution of hydroxystearic acid in liquid petrolatum. If the amount of the acid solution was small, the liquid petrolatum solution was also warmed. Upon cooling, a jellified product results; agitation is not necessary if the material is soluble in the liquid petrolatum. If the material is insoluble, as for example, zinc oxide, agitation is necessary while cooling to prevent separation of the solid. If desired, the medicament may be incorporated with the solidified jelly prepared by warming the liquid petrolatum and adding the acid solution to this. A stock jellified liquid petrolatum may be prepared and used in the same manner as white petrolatum. The following examples are chosen to illustrate pharmaceutical compatibility and are not chosen for therapeutic fitness.

Camphor Ointment: camphor 22 Gm., liquid petrolatum 72 Gm., 20% acid 6 Gm.

Capsicum Ointment: oleoresin of capsicum 5 Gm., petrolatum 85 Gm., 20% acid 10 Gm.

Compound Menthol Ointment: menthol 10 Gm., methyl salicylate 10 Gm., liquid petrolatum 65 Gm., 20% acid 15 Gm.

Ichthammol Ointment: ichthammol 10 Gm., liquid petrolatum 80 Gm., 20% acid 10 Gm.

Mustard Ointment: volatile oil of mustard 2 Gm., liquid petrolatum 88 Gm., 20% acid 10 Gm.

Phenol Ointment: phenol 2 Gm., liquid petrolatum 88 Gm., 20% acid 10 Gm.

[1] Manufactured by National Oil Products Co., Harrison, New Jersey.

[2] Courtesy of L. Sonneborn Sons Inc., New York.

Table II.—Jellified Mineral Oils

Oil No.	% Acid	Consistency[a] 100 Gm.	200 Gm.	300 Gm.	400 Gm.	500 Gm.	1000 Gm.
1	4	0	0	tr	0.5	1.0	+
1	3	0	0	0.5	5.0	+	
1	2	tr	4.0	9.0	+		
1	1	0.5	+				
2	4	0	0	tr	1.0	3.0	+
2	3	0	0.5	2.0	10.0	+	
2	2	0.5	7.0	+			
2	1	2.0	+				
3	4	0	0	0.5	1.5	5.0	+
3	3	0	0.5	3.5	14.0	+	
3	2	0.5	10.0	+			
3	1	+					
4	4	0	0	1.0	3.0	10.0	+
4	3	0	0.5	4.0	18.0	+	
4	2	1.0	13.0	+			
4	1	+					
5	4	0	0	1.5	3.5	12.0	+
5	3	0	1.0	8.0	+		
5	2	3.0	+				
5	1	+					
6	4	0	0	2.0	8.0	+	
6	3	0.5	2.0	11.0	+		
6	2	3.0	+				
6	1	+					
7	4	0	2.0	3.0	13.0	+	
7	3	2.0	8.0	+			
7	2	7.0	+				
7	1	+					
8	4	1.0	3.0	5.0	15.0	+	
8	3	3.0	18.0	+			
8	2	9.0	+				
8	1	+					

[a] Mm. penetration in 60 seconds with weight noted: "tr" indicates trace; "+" indicates penetration to the bottom of the ar before 60 seconds interval.

Scarlet Red Ointment: scarlet red 5 Gm., liquid petrolatum 85 Gm., 20% acid 10 Gm.

The above ointments are, in the most case, materials which are more or less soluble in liquid petrolatum. The base is also satisfactory for substances which are insoluble as indicated by the following examples:

Ammoniated Mercury Ointment: ammoniated mercury 10 Gm., liquid petrolatum 80 Gm., 20% acid 10 Gm.

Boric Acid Ointment: boric acid 10 Gm., liquid petrolatum 70 Gm., 20% acid 20 Gm.

Sulfur Ointment: precipitated sulfur 15 Gm., liquid petrolatum 72.5 Gm., 20% acid 12.5 Gm.

Zinc Oxide Ointment: zinc oxide 20 Gm., liquid petrolatum 70 Gm., 20% acid 10 Gm.

Cosmetics.—The following are examples of cosmetics prepared using hydroxystearic acid as a jellifier.

Cold Cream

Stearic acid	4 Gm.
Spermaceti	4 Gm.
White wax	6 Gm.
20% Acid	25 Gm.
Liquid petrolatum	75 Gm.
Water	50 Gm.
Triethanolamine	2 Gm.

Melt the stearic acid, spermaceti, white wax and 20% acid in the order named, add the mineral oil, continue heating until a solution results. Dissolve the triethanolamine in the water and heat to the same temperature as the oil solution. Mix with a mechanical agitator until emulsified; stir occasionally while cooling. This cream differs from most creams in that it has a soft, jelly-like consistency.

Liquefying Cold Cream

20% Acid	50 Gm.
Liquid petrolatum	50 Gm.
Water	60 Gm.
Triethanolamine	2 Gm.

Heat the acid with the liquid petrolatum. Dissolve the triethanolamine in the water; heat to the same temperature as the oil solution. Mix with a mechanical agitator, and stir occasionally until cool. This cream readily liquefied when applied to the skin.

Liquefying Cream (Not Emulsified)

Spermaceti	5 Gm.
White wax	5 Gm.
20% Acid	20 Gm.
Liquid petrolatum	70 Gm.

Melt the spermaceti, white wax and 20% acid; add the liquid petrolatum, warming if necessary to effect solution. Stir until partially cool. This mixture readily melts when applied to the skin.

Brilliantine

20% Acid	17.5 Gm.
Liquid petrolatum	82.5 Gm.

Melt the 20% acid and incorporate with the liquid petrolatum, warming if necessary to effect solution. Allow to stand without agitation. A liquefying gel results.

Other Jellified Products.—Liquefying gels were produced when hydroxystearic acid was dissolved in other substances. Fixed oils, such as almond, linseed, cottonseed and sesame, produced gels with about 2.5% of hydroxystearic acid. Hydrocarbons such as kerosene and benzene produced transparent gels with 10% of the acid. Other hydrocarbons, benzene and toluene, required more hydroxystearic acid and produced a more opaque, somewhat granular appearing product. Gels were also produced with 10% of the acid in volatile oils such as turpentine and methyl salicylate.

SUMMARY

Hydroxystearic acid was shown to be a jellifying material for liquid petrolatum and fixed oils in quantities of 1 or 2 per cent. In larger quantities it also produced gels with hydrocarbons and volatile oils.

Liquefying ointments were prepared using jellified liquid petrolatum to replace petrolatum of the U. S. P. and N. F. formulas. The base appeared to be compatible with medicaments in the same manner as petrolatum and produced ointments which readily liquefied when placed upon the skin. The consistency of the final ointment varied with the amount of hydroxystearic acid present.

Used in cosmetics, the addition of hydroxystearic acid definitely improved the consistency of products containing liquid petrolatum, whether solutions or emulsions.

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Book Review

The Chemist's Dictionary of Synonyms, incorporating Rouse's Synonyms. Published at the Office of the Chemist and Druggist, 28 Essex Street, Strand, London, W. C. 2, 1940. 6 x 9, 136 pages. Price, postpaid, 5s., 4d.

The purpose of the publication is to supply definitions of terms for quick reference in the pharmacy and while the book is intended for the British pharmacy, it has related value for all pharmacists. Very likely British synonyms will be brought to the attention of American pharmacists more frequently because of war conditions.—E. G. E.

The Most Comprehensive Food and Drug Bill

Introduced into the United States Congress by William H. F. Lee (May 31, 1837, to October 15, 1891) Soldier, Agriculturist and Legislator

*By Lyman F. Kebler**

Of the more than one hundred food and drug bills introduced into the United States Congress, over a period of 25 years, that finally resulted in the enactment of the National Food and Drugs Act, June 30, 1906, Representative Lee's bill (H. R. 10320), introduced (1) June 4, 1888, is considered by many the most comprehensive. This is the first of the modern food and drug bills introduced into Congress by a Representative of Virginia. Congressman R. L. T. Beale of Virginia, a member of the Congress that enacted the Drug and Chemical import law in 1848, over thirty years later, introduced the second food bill in 1879 and Representative John S. Barbour of Virginia, introduced a food bill for the District of Columbia in 1886. Neither of the two latter bills covered drugs.

BIOGRAPHICAL SKETCH OF AUTHOR OF BILL

Representative William H. F. Lee was born in the Lee Mansion, just across the Potomac River from the Lincoln Memorial, the second son of the illustrious General Robert E. Lee and Mary Ann Randolph (Custis) Lee, the granddaughter of Martha Washington, by her first husband. This is another instance of a Lee marrying into a wealthy, prosperous, influential family. A study of these marriages forces the conclusion that the blending of the blood and fortunes of some of these wives with the Lees were distinct assets to successive generations. There seemed to be something in the female side that inspired their sons to greater endeavors.

One of objects of some of the marriages in those days was, in part at least, the combining of fortunes and families of influence. It should be noted that the oldest son inherited the major part, if not all of the family estate and that while the younger brothers usually inherited comparatively little, some of them increased it by winning the hands of wealthy heiresses. How well some of them succeeded is shown by a study of the history (2) and

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genealogy of the Virginia Lees. Even though the daughters came in second in the matter of property inheritance, when there was a brother, the Lee sons seemed to find and win the companionship of wealthy, noble-blooded women. This subject could be profitably expanded, but it would lead us too far afield.

As the result of financial embarrassment, the necessity of educating his children in the good public schools in Alexandria, Virginia, the grandfather of William H. F. Lee moved from the beautiful Stratford Hall into modest quarters in Alexandria. Free schools were supported by taxes (3) in Virginia as early as 1739, and would probably have come earlier had not Governor Berkeley expressed opposition to them. The foundation of the Alexandria Academy was laid by the Freemasons in 1760. It was in these schools that Robert E. Lee received his early education, which was finished at West Point. He was not considered a secessionist. It was in the Alexandria schools that Robert's children obtained at least a part of their early education. It must be said, however, that information regarding their early schooling is rather fragmentary.

PROPERTY INHERITANCE AND SOME OF THE VICISSITUDES IN "ROONEY" LEE'S LIFE

By a complicated will of her father Mrs. Lee was given a life interest in the Lee Mansion and the Arlington tract of 1100 acres in 1857. This property on her death was to go to her oldest son, but the Civil War complicated matters. By the same will, William H. F. Lee, familiarly known as "Rooney," was left the "White House" plantation, of 4000 acres, near Richmond, Virginia. "Rooney's" academic education was obtained in Harvard University with the class of 1857. While at Harvard he is alleged to have shown the "Virginia habit of command," to which he was born and bred. He was a powerfully built man, six feet and two inches tall, pulled the stroke oar on the Harvard crew and was considered one of the best oarsmen at the time. In 1857 he was appointed second lieutenant in the United States Army, resigned his commission in 1859 and became a farmer on his "White House" estate. During the same year he married his cousin, Charlotte Wickham. At the outbreak of hostilities, between the States, being an ardent Confederate, he organized a picked cavalry, was appointed captain of cavalry and gradually was promoted to the rank of Major General as the war continued. His military career is recorded in biographies (4) and need not be considered here in detail. Suffice it to say, he was considered an efficient officer, an exemplary soldier and highly regarded by his men. He was wounded, captured, interned in Fortress Monroe, transferred to Fort Lafayette, kept as a hostage in solitary confinement, because certain United States officers were being held under sentence of death in Libby Prison. If these officers were executed, it was intended that General Wm. H. F. Lee should suffer the same fate. Fortune intervened. The General was exchanged in March 1864. During his imprisonment his beloved wife and two children passed on.

The impression seems to prevail that there was great suffering, as is the rule in most wars, in the Southern States, because of drouths and a shortage of labor. The General probably saw his share. But statisticians tell us that there was plenty of food. The difficulty was to get the food to the places where it was needed.

After the close of the war "Rooney" returned to his "White House" estate, which was not confiscated, but he found his fine home in ashes. He there resumed agricultural pursuits until 1874, when he transferred his activities to the Ravensworth plantation, about ten miles from Alexandria, Virginia. In 1867, he married Mary Tabb Bolling, who with two children survived him. He served as President of the Virginia State Agricultural Society.

In 1876, he was elected to the Virginia Senate, took his seat (5) December 6th the same year and served until April 2, 1879. He also acted as Chairman of the Senate on occasions. He was elected three times as Representative to the United States Congress and served from 1887 to the time of his death 1891.

TITLE AND SOME OF THE SALIENT FEATURES OF THE LEE FOOD AND DRUG BILL

The title of the Lee Food and Drug bill (6) reads: "To prevent the sale, manufacture, or transportation of adulterated articles of food, drink, and drugs manufactured or made in one State and intended to be sold in another, or intended for export or for sale in the District of Columbia or any Territory or Reservation of the United States, and to prevent the exportation or importation of adulterated food, drink, or drugs, and for creating a pure food division in the Department of Agriculture."

It is one of the most comprehensive titles of any food and drug bill ever introduced into Congress, and if the term misbranded, of which Representative Lee is apparently the author, had been included and the language construed as of our own time, most adulterations, unfair methods of exploitations, untruthful claims and representations, including imports and exports, made for the foods, drinks and medicines covered, could have been controlled. The Lee bill was one of sixteen food and drug bills introduced into Congress during the first term of President Grover Cleveland. It followed

close on the heels of the enactment of the Oleomargarin Law and the hearings of the Agricultural Committee on lard during the latter of which some awful conditions were disclosed. The bill was referred to the Committee on Agriculture. Because of the conditions disclosed during the lard hearings no hearings were held on this bill. The bill is unique in the phraseology used and the number of features covered.

This bill embodies the designation "Pure Food" and, as defined, covers all articles of diet, drinks and drugs consumed for sustaining life or restoring health, or for beverage or medicinal purposes. This phrase was commonly used and is still used to cover food and drug legislation in general. The same idea is embodied in the phrases "Food Division" and "Food Inspectors" used in the bill.

The artificial coloring or flavoring or otherwise manipulating liquors, whether for beverage or medicinal use, so as to deceive the purchaser or consumer, as to age, source or otherwise, is prohibited. The phrase "Pure Drugs" includes all medicines recognized by the United States Board of Health, those covered by the United States Pharmacopœia and other pharmacopœias, recognized by this Board, for either internal or external use. The creation of this Board (7) was authorized by Congress in 1879.

The interstate shipment or importation of illegal foods, drinks or medicines is interdicted. Transportation companies are adjudged guilty of a misdemeanor for carrying adulterated or misbranded foods, drinks or drugs into interstate commerce. A fine of not less than $100 nor more than $1000 could be imposed by the court for each offense and imprisonment for a second conviction. All things considered, these were rather stiff penalties. Specific coverage of transportation companies was another new feature in this type of legislation. Later laws were passed covering the transportation of certain drugs and meats and meat food products.

Lee's bill is the first food and drug bill that contains the term "Misbranded." The elements of "Intent" and "Knowingly" do not find a place in this bill. There was much contention as to what branch of the Government should be vested with the enforcement of this type of legislation. Congressman Lee was essentially an agriculturalist and naturally favored the Department of Agriculture. Indeed, his bill is the first that designates this Department as the enforcing agent. Provisions are made for the creation of a "Food Division" in this Department, to be supervised by a Chief who, like the Chemist, is to be appointed by the President. The Chemist is directed to make rules and regulations and establish uniform methods of analysis. Assistant chemists are to be appointed in fourteen of the large cities, named in the bill.

The bill authorizes the President to appoint one "Food Inspector" for each state and territory and the District of Columbia. These inspectors are given extensive and responsible duties, namely: the sampling of articles covered by this bill, having same verified and analyzed by the chemists; "inspect slaughter-houses and have microscopic and analytical tests made of slaughtered animals, and when they find adulterated articles of food, drink, or drugs, or tainted or diseased meat sold or offered for sale in violation of this act, they shall present the facts properly verified under oath to the district attorney, who shall immediately proceed to prosecute all violators of this act in the courts of the United States." The bill also provides for giving half of a fine to any informant whose information leads to a conviction, but he must first of all give suitable bond to cover expenses in case of failure to convict and, moreover, if there is any evil motive, he is liable to have the costs of the trial assessed against him by the court. Furthermore, any person or association was at liberty to lay any information he or it may possess before any state inspector, a United States district attorney, or a United States grand jury, but in the latter cases no part of any fine shall inure to the informant.

Any food, drink, drug or medicine or admixture or compound of the same, recognized by the National Board of Health and not deleterious to health, licensed by the

Chief of the "Pure Food Division," may be manufactured, sold or transported without any penalty of this act, provided that they are "properly designated and branded as prescribed by the rules of the said Pure Food Division, so as to give the purchaser a correct idea of what he is purchasing." The granting of a license required the payment of a registration fee not to exceed $10.00 for each article. The language of the act in connection with the licensing and registering, were construed to give the "Food Division," the power to require manufacturers and dealers to give the consumer the composition of such articles. These requirements were not kindly received by the trade. The time was not ripe for this advanced step.

A companion bill (S. 3217) to the Lee bill was introduced into the Senate by Senator Henry W. Blair of New Hamshire, June 25, 1888. It died in committee.

ACTION ALONG NEW LINES STARTED BY LEE BILL

Even though the Lee bill was considered constitutionally sound, provided for honesty, fair dealing and the general welfare and it was believed that it would curb practices threatening our national health, the Committee on Agriculture, in its report (8), through its chairman, James Laird, recommended that the Lee bill be laid on the table and that the Committee's substitute bill (H. R. No. 11266) be passed. The substitute bill clarified several of the features embodied in the Lee bill. It requires the ingredients of compounded foods and drugs to be declared on the labels; provides for the inspection of live stock prior to slaughtering and the inspection of meats and meat food products thereof by veterinarians; requires suitable marking, labeling or stamping of meats and meat food products; prohibits the shipping into interstate commerce of uninspected, unlabeled, untagged or unstamped carcasses or meat or meat foods and requires manufacturers of foods and drugs to take out a Government license each, at a fee of $10.00.

Due probably to the active interest displayed in this bill by Congress, its far-reaching effects and the public demands for this kind of legislation, tremendous opposition thereto was aroused in various quarters. Among the features particularly obnoxious were the inspection of live stock, meats and meat food products, requiring a declaration on the label of the ingredients of compounded foods and drugs and the procuring of a Government license. Most of the features of the Lee bill and its substitute were in due time enacted into federal laws; among them may be mentioned the Reprisal Act (9) 1890; the 1891 Appropriation Act of the Department of Agriculture (10); the Serum, Virus Act of 1902 (11); the Meat Inspection Act (12) and the Food and Drug Act, both of 1906 (13) and the Food, Drug and Cosmetic Act of 1938 (14).

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Book Review

Vitamin E. A symposium held under the auspices of the Society of Chemical Industry. viii + 88 pages, 5½ x 8½. 1940. New York: Chemical Publishing Company, Inc. $2.00.

The book brings out facts on the chemical structure, chemical properties, physiological action, effects of deficiency of and clinical uses of vitamin E. The monograph is an informative treatise on this vitamin discovered in 1922 and about which comparatively little is yet known. A valuable feature is that it contains 165 references to the literature.—A. G. D.